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13. Abstract

Poly(ADP-ribose) polymerase (PARP) has strong affinity for DNA strand breaks and cycles on and off the DNA ends to allow DNA repair. A DNA-binding domain of PARP (PARP-DBD) acts as a dominant-negative mutant by binding to DNA strand breaks irreversibly and sensitizing mammalian cells to DNA-damaging agents. Therefore, expression of PARP-DBD in prostate carcinoma cells offers a strategy to achieve sensitization to genotoxic treatments. Toward this end, we developed recombinant plasmids expressing the PARP-DBD under the control of the 5'-flanking sequences of the human prostate-specific antigen (PSA) gene. Tissue specificity of PARP-DBD expression in human tumor cells was confirmed using the PSA-producing (LNCaP) and PSA-negative (PC-3) prostate cancer cells, as well as cells of non-prostate origin, Ewing's sarcoma (A4573 cells). LNCaP cells stably transfected with the PSA-regulated cDNA for PARP-DBD exhibit an androgen-dependent induction of PARP-DBD expression as determined by Western blotting, RT-PCR and *in situ* immunofluorescence. Further, we found that PARP-DBD sensitized LNCaP cells to DNA-damaging agents, such as ionizing radiation and etoposide. Androgen (R1881)-dependent stimulation of PARP-DBD expression resulted in a two-fold growth inhibition in LNCaP cells as compared to controls, and an augmented apoptotic cell death in response to ionizing radiation or etoposide. Taken together, the plasmid vector developed in this study permits the expression of the human PARP-DBD in an androgen-inducible and PSA-dependent fashion and sensitizes prostatic adenocarcinoma cells to DNA-damaging treatments. These results provide proof-of-principle for a novel therapeutic strategy for the treatment of prostate cancer.

14. SUBJECT TERMS:
prostate cancer, poly (ADP-ribose) polymerase, radiation, DNA damage

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FINAL REPORT

INTRODUCTION

The central objective of the proposal is to express the DNA-binding domain of PARP under control of prostate tissue-specific promoter in prostate cancer cells and sensitize them to radiotherapy or chemotherapy. We hypothesize that the sustained presence of the PARP-DBD in prostate tumor tissue will kill cells via apoptosis in response to massive DNA damage induced by ionizing radiation or genotoxic drugs. To test this hypothesis we will utilize the prostate-specific antigen (PSA) promoter to direct the PARP-DBD expression to prostate cancer cells. Using PSA-producing cells (LNCaP) and cells that do not express PSA (PC-3) as the primary experimental model system we propose the experimental approach designed to: 1) produce prostate carcinoma cell sublines which allow androgen-inducible, high-level expression of the PARP-DBD and 2) test the DNA-binding domain of PARP as a molecular sensitizer for improving responses of prostate tumor cells to gamma radiation and DNA-damaging drugs. The completion of experiments proposed in this project will contribute to the development of complementary biotherapeutic approaches in the treatment of prostate cancers, which fail local-regional therapy.

I. ORIGINAL STATEMENT OF WORK

The proposed studies are designed to explore the potential of novel combination therapy that would utilize the tissue-specific (prostate) and radiation-specific (damages in DNA) gene therapy for prostate cancer.

Task 1. To establish prostate cancer cell lines stably expressing PARP-DBD under control of PSA promoter regulatory elements (months 1-19)

i. develop a series of plasmids to drive prostate tissue-specific expression of PARP-DBD gene (months 1-8)
ii. produce PARP-DBD expressing sublines from LNCaP prostate carcinoma cell line (months 9-13)
iii. test tissue-specificity and responsiveness of PARP-DBD expression to androgens (months 14-19)

Task 2. To investigate the potential of PARP-DBD protein for sensitization of prostate cancer cells to ionizing radiation and DNA-damaging drugs (months 19-36)

i. test the PARP-DBD expression levels for efficiency to inhibit PARP activity and DNA damage repair following gamma radiation and drug treatments (months 19-24)
ii. investigate the effects of PARP-DBD expression on cell viability, cycle progression and apoptosis induction post-irradiation (months 24-31)
iii. determine whether cell sensitization by PARP-DBD depends upon the type of DNA damage inflicted on the cells (months 26-32)
iv. conduct radiation survival curve analysis on prostate cancer cell lines expressing differential levels of PARP-DBD to assess its radiosensitizing ability (months 28-36)

II. RESEARCH ACCOMPLISHED

Task 1. To establish prostate cancer cell lines stably expressing PARP-DBD under control of PSA promoter regulatory elements

Human PSA promoter/enhancer drives expression of the PARP-DBD in LNCaP cells.

This study is focused on the unique properties of the DNA-binding domain of PARP as a potent molecular sensitizer to DNA damaging treatments (1,2). We isolated and cloned the fragment of human PARP cDNA encompassing the region (aa 1-234) that encodes two zinc fingers of the enzyme as well as the KKKSKK nuclear localization signal (PARP-DBD). Subsequently, we developed plasmid vectors to express human PARP-DBD as a Flag-fusion protein in human prostatic adenocarcinoma cells (LNCaP cell line) both constitutively and in androgen-dependent fashion (Fig. 1A). The recombinant plasmid, pCMV-DBD/F, permits constitutive expression of the PARP-DBD under control of the human CMV promoter. To achieve tissue-specific expression of the PARP-DBD in the androgen-sensitive LNCaP cells, we have constructed an expression vector, pPSA(e/p)-DBD/F, comprised of the coding region for the DNA-binding domain of PARP linked to 5'-flanked sequences (1.3 kb upstream enhancer – 0.6 kb minimal promoter) of the human PSA gene. The expression of the PARP-DBD Flag-fusion protein in LNCaP cells was confirmed in transient transfection assays (Fig. 1B). Immunoblot analysis of cell lysates revealed that exogenous PARP-DBD Flag fusion protein has a molecular mass 31 kDa consistent with the length of corresponding cDNA, and is recognized by anti-Flag and anti-PARP antibodies. Functional activity of expressed PARP-DBD Flag-fusion protein was assayed in DNA binding reactions using double stranded 5'-biotinylated oligonucleotides coupled to streptavidin-coated magnetic beads. These beads were used to recover DNA binding proteins from LNCaP cells transiently transfected with pCMV-DBD/F plasmid. We found (Fig. 1C) that both endogenous PARP and PARP-DBD fusion protein are captured effectively by DNA fragments, thus indicating that PARP-DBD retain its DNA-binding activity when expressed in LNCaP prostate carcinoma cells.

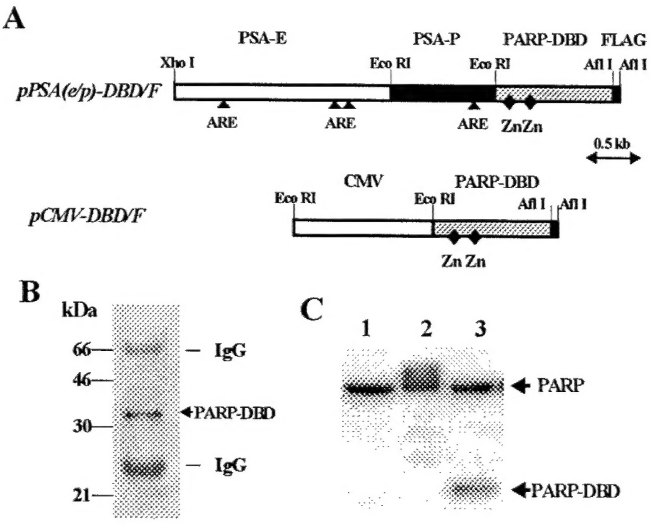


Figure 1. **A:** A schematic representation of the recombinant constructs for constitutive (pCMV-DBD) and androgen-inducible (pPSA-DBD) expression of the human PARP-DBD in prostate cancer cells. PSA-E, 1.3 kb upstream PSA enhancer region; PSA-P, 0.6 kb minimal promoter of the human prostate-specific antigen (PSA) gene. Relevant restriction enzyme sites, zinc fingers (Zn) and androgen response elements (ARE) are indicated. **B:** Immunodetection of the PARP-DBD in LNCaP cells transiently transfected with pPSA(e/p)-DBD/F. **C:** Detection of DNA-binding activity of wild-type PARP and PARP-DBD Flag-fusion proteins. Purified recombinant PARP (1) and cell extracts prepared from parental LNCaP cells (2) or LNCaP cells, transiently transfected with the pCMV-DBD/F (3), were bound to 5'-biotinylated double-stranded oligonucleotides coupled with streptavidin-coated magnetic beads. Proteins were eluted and analyzed by Western blotting using polyclonal

anti-PARP antibodies as described in manuscript (Cancer Research, 2002, 62: 6879-6883) included in **appendix**.

Tissue specificity and androgen responsiveness of the PARP-DBD expression in LNCaP cells.

The 5'-regulatory sequences of the human PSA gene have been cloned and characterized (3,4). Deletion analysis of this region identified a minimal (core) promoter region (nt -320 to +12), strong upstream enhancer (nt -5824 to -3738) and the presence of down-regulating elements within the central region (nt -4136 to -541). Previous studies have identified the 5'-enhancer element linked to minimal core promoter of the human PSA gene as an effective combination of regulatory elements capable of driving the expression of reporter genes in PSA-producing prostate cancer cells both *in vitro* and *in vivo* (5,6). Consequently, we developed and tested the construct, pPSA(e/p)-DBD/F, for its ability to drive the expression of PARP-DBD in a tissue-specific fashion, and its androgen responsiveness in prostate carcinoma cells.

Tissue specificity of PARP-DBD expression under control of the PSA promoter/enhancer was evaluated in transient transfection assays using the PSA-producing (LNCaP) and PSA-negative (PC-3) prostate cancer cells, as well as cells of non-prostate origin such as Ewing's sarcoma (A4573 cell line). We found that PSA enhancer/promoter-driven expression of the human PARP-DBD was immunodetectable observed only in PSA-producing LNCaP prostate carcinoma cells but not in PSA-independent cell lines (Fig. 2). Although more PSA-producing cell lines need to be tested to elaborate a PSA-dependence of PARP-DBD expression, our data are consistant with previously reported findings that PSA promoter retains its tissue specificity both *in vivo* and *in vitro* (5,6).

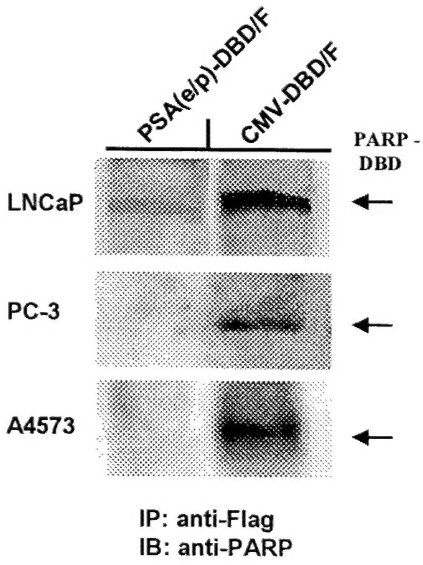
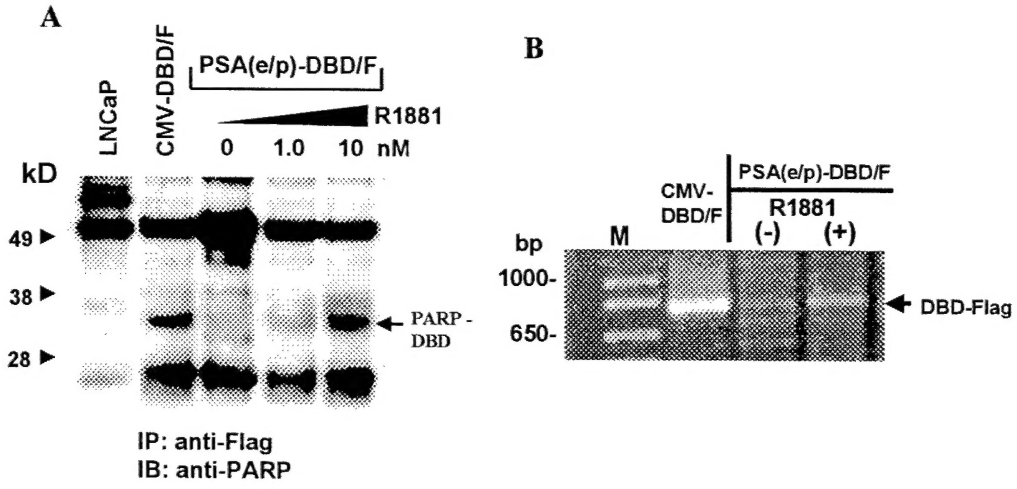


Figure 2: PARP-DBD expression in PSA-producing and PSA-negative cells. PSA-positive (LNCaP), PSA-insensitive (PC-3) prostate cel l lines, and non-prostate (Ewing's sarcoma, A4573 cell line) cells were transiently transfected with pPSA(EP)–DBD/F or pCMV-DBD/F. Cells were harvested 48 hours after transfection and PARP-DBD expression was immunodetected.

The 5' flanking region of the human PSA gene contains several androgen-responsive elements and is responsible for the androgen-dependent expression of PSA in benign and malignant prostate cells. To evaluate whether the PSA promoter / enhancer constructs support androgen responsiveness of PARP-DBD expression, LNCaP carcinoma cells were stably transfected with PARP-DBD expression vectors and established polyclonal LNCaP sublines (PSA-

DBD and CMV-DBD) were subsequently subjected to analysis of PARP-DBD expression levels. Cells were grown in media containing charcoal-stripped serum for seven days followed by incubation for 24 hours in the absence or presence of the synthetic androgen, R1881 (0-10 nM). Western blot analysis and RT-PCR were performed to evaluate androgen-regulated expression of the human PARP-DBD in LNCaP cells (Fig. 3). Parental LNCaP cells and the LNCaP cell subline (CMV-DBD) were used as negative and positive controls, respectively, for PARP-DBD expression levels in these experiments. We found that exposure of PSA-DBD cells to androgen (R1881) resulted in dose-dependent stimulation of PARP-DBD expression at levels of mRNA (Fig. 3B) and protein (Fig 3A). No notable changes in the PARP-DBD expression levels have been observed in control cell lines exposed to R1881 at doses up to 10 nM.

Figure 3: LNCaP cells were stably transfected with plasmid vectors that allow constitutive (pCMV-DBD/F) or androgen-inducible (pPSA-DBD/F) expression of PARP-DBD. The established cell sublines



were analyzed for androgen-dependent induction of the PARP-DBD expression by Western blotting and RT-PCR. **A**, Immunodetection of PARP-DBD-Flag fusion protein in LNCaP cell sublines expressing PARP-DBD under control of CMV promoter (CMV-DBD) or PSA enhancer /promoter (PSA-DBD). LNCaP sublines transfected with pPSA-DBD/F were maintained in absence or in presence of synthetic androgen, R1881 (0-10 nM). Parental LNCaP cells were used as a negative control for PARP-DBD expression. The migration of the DBD-Flag fused protein is indicated on the right. **B**, RT-PCR analysis of mRNA for PARP-DBD –Flag fused protein. Specific RT-PCR product is indicated on the right, and molecular weight markers (M) are shown on the left.

Androgen-dependent regulation of PARP-DBD expression in PSA-DBD prostate carcinoma cells was further confirmed by *in situ* immunodetection of the PARP-DBD-Flag fusion protein using fluorescence microscopy (Fig. 4). These data indicate that the pPSA(e/p)-DBD/F recombinant vector allows expression of functionally active DBD of PARP *in vitro*, and that the androgen-dependent expression is specific to PSA-producing prostate carcinoma cells.

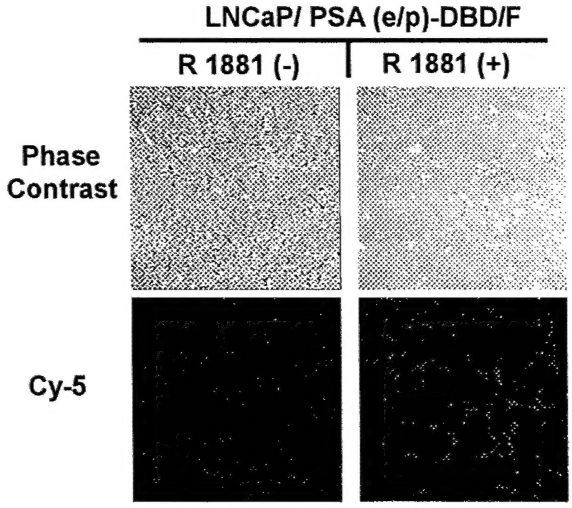


Figure 4: pPSA-DBD/F drives androgen-responsive expression of PARP-DBD in LNCaP cells. For *in situ* PARP-DBD immunodetection, LNCaP cells were grown in media containing 10% charcoal-stripped fetal bovine serum for seven days. Following induction of PARP-DBD expression by synthetic androgen R1881 (10nM) for 24 h, cells were immunostained for PARP-DBD-Flag fusion protein. Transmitted (phase contrast) and Cy5 (red fluorescence) images were acquired using IX 70 confocal laser scanning microscope (Olympus).

Task 2. To investigate the potential of PARP-DBD protein for sensitization of prostate cancer cells to ionizing radiation and DNA-damaging drugs

PARP-DBD expression sensitizes LNCaP cells to DNA-damage.

The PARP-DBD fragment acts as a *trans*-dominant inhibitor of PARP activity by competing with endogenous wild-type PARP for DNA strand breaks (1,2). Furthermore, using atomic force microscopy we have recently demonstrated that PARP-DBD binds to broken DNA strands irreversibly, making them inaccessible to DNA repair enzymes (7). These data suggest that forced expression of the PARP-DBD can impair the function of endogenous PARP in cellular responses to DNA damage leading to accumulation of sustained lesions in the genome, thereby overcoming cellular resistance to radio- and chemotherapeutic intervention. In support of this suggestion, the sensitization of the DBD-expressing mammalian cells to ionizing radiation and DNA-damaging agents has been recently demonstrated (1,2).

To investigate whether PARP-DBD would increase the susceptibility of human prostate carcinoma to DNA-damaging treatments, the expression of PARP-DBD in LNCaP (PSA-DBD) cells was induced by R1881, and cells were subsequently exposed to ionizing radiation or etoposide (VP-16). We found that androgen (R1881)-dependent stimulation of PARP-DBD expression significantly enhanced (at least a two-fold) growth inhibition of PSA-DBD cells in response to DNA damage, compared to control cells (Fig. 4A). This effect appeared to be strictly related to the PARP-DBD expression in LNCaP cells and cannot be accounted for the presence of androgen in the growth medium. In fact, other studies have shown that androgens are potent stimulators of LNCaP cells growth *in vitro* (8).

We next examined whether the PARP-DBD - mediated sensitization of LNCaP cells to DNA damage is attributable to an increased rate of apoptosis. Quantitative measurements of cell death were carried out using Annexin V- propidium iodide staining and mitochondrial depolarization assays. Previous studies show that LNCaP cells are highly resistant to ionizing radiation, and fail to activate classical apoptotic pathways in response to DNA-damaging treatments (9). In agreement with these findings, we found that parental LNCaP cells as well as

the un-induced PSA-DBD cell subline exhibit only marginal levels of cell death after exposure to ionizing radiation or etoposide (Fig. 4). When PARP-DBD expression was induced by R1881, irradiated or etoposide-treated LNCaP (PSA-DBD) cells showed significantly (more than two fold) increased staining for Annexin V (Fig. 4B) and depolarization of mitochondrial membrane (Fig. 4C) within 24 hours of treatment.

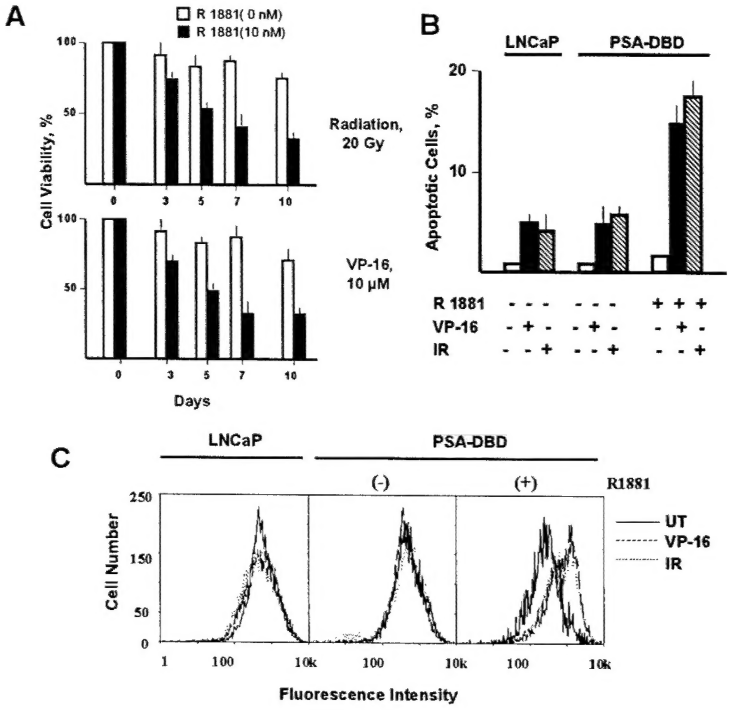


Figure 5: PARP-DBD sensitizes human prostate cancer cells to ionizing radiation and etoposide. **A:** PARP-DBD expression enhances DNA damage-induced growth inhibition in prostate carcinoma cells. LNCaP (PSA-DBD) cells were maintained in media containing 10% charcoal-stripped fetal bovine serum in presence (black columns) or absence (open columns) of synthetic androgen R1881 (10 nM) prior to irradiation (20 Gy) or treatment with etoposide (10µM). Viable cells were measured by an MTS assay at indicated times and results are expressed as a percentage of mock-treated control (n=4). Standard deviations from three independent experiments are indicated. **B:** Effect of PARP-DBD expression on annexin staining in LNCaP cells following to DNA-damaging treatments. PSA-DBD cells were maintained in absence or in presence of R1881 (10 nM) for 24 h prior to irradiation (IR; 20 Gy) or treatment with etoposide (10µM; VP-16). Annexin V binding activity was determined in parental LNCaP and PSA-DBD cells by Flow cytometry 24 h after treatments. Apoptotic cells are defined as Annexin V positive cells and are expressed as percentage of total cell number in sample analyzed on FACSscan flow cytometer. Data presented are the mean values determined from triplicate experiments. **C:** Effect of PARP-DBD expression on changes of mitochondrial membrane potential in LNCaP cells following to DNA-damaging treatments. 24 h after treatments, untreated controls (UT), irradiated (IR; 20 Gy) or etoposide-treated (10µM; VP-16) cells were stained with JC-1 "DePsipher" reagent and analyzed by flow cytometry. Mitochondrial potential breakdown in dying cells results in accumulation of green fluorescent JC-1 monomers, which, in turn, is reflected by an increase of green fluorescence events. Representative data (of three independent experiments) are shown.

These data indicate that perturbation of PARP function via enforced expression of its dominant negative mutant (PARP-DBD) results in enhanced sensibility of prostate cancer cells to DNA damaging treatments. Considering the fact that androgens block apoptosis in LNCaP cells

triggered by diverse agents, including ionizing radiation (10), our observations suggest that over-expression of the PARP-DBD augments apoptotic pathways in these cells in an androgen-independent fashion. Additional investigations are required to further elucidate the mechanisms for PARP-DBD–mediated sensitization of LNCaP cells to DNA damage, as well as the enhanced apoptotic responses in DBD-expressing prostate cancer cells. The studies addressing these questions are currently underway.

PARP-DBD and DNA-damage response.

The study was initiated to investigate the role of PARP-DBD in cellular responses to ionizing radiation and DNA damaging treatments. Recent studies have also implicated PARP in transcription of eukaryotic genes (11-13). To elucidate mechanistical basis for PARP role in transcription, we investigated whether PARP can be recruited to gene-regulating sequences and whether its DNA-binding activity has a role in PARP-mediated gene regulation. Based on PARP ability to interact with partially unwound DNA (14), we reasoned that DNA secondary structures with single-stranded character may provide potential binding sites for PARP in gene regulating sequences in the absence of DNA strand breaks. In this work we investigated the interactions between PARP protein and DNA structures of different complexity such as DNA heteroduplexes carrying stable secondary structures and superhelical DNA containing PARP promoter sequences. We found that PARP can recognize non-canonical conformations (hairpins) in a DNA end-independent fashion, and it is capable of *in vitro* binding to the PARP promoter sequences where the dyad symmetry elements may form the cruciforms. Using a chromatin cross-linking and immunoprecipitation assay we show that the human PARP promoter is an *in vivo* target for PARP protein. Further, we show that PARP protein down-regulates its gene promoter, and that DNA-binding activity of PARP is essential for its function in transcription (Fig. 6).

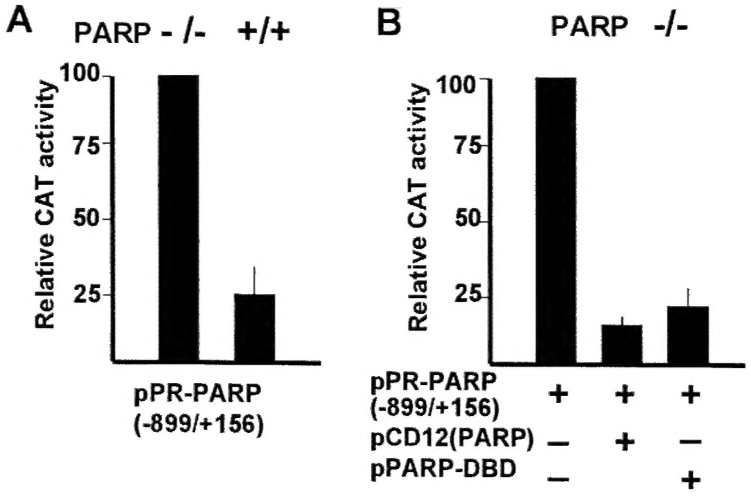


Figure 6. PARP protein is a transcriptional repressor. **A**, PARP promoter (pPR-PARP) transcriptional activity in wild-type (PARP $^{+/+}$) and PARP $^{-/-}$ fibroblasts. **B**, expression of human PARP or its DNA-binding domain down-regulates promoter activity. PARP $^{-/-}$ cells were co-transfected with pPR-PARP and plasmids encoding for full length PARP (pCD12) or its truncated mutant (pPARP-DBD/F). CAT activity of pPR-PARP in PARP $^{-/-}$ cells was arbitrarily taken as 100%. Means of triplicate experiments normalized by co-

transfected β-gal and standard deviations are indicated. Experimental procedures for these experiments are described in manuscript (J. Biol. Chem., 2002, 277: 665-670) included in **appendix**.

Our data suggest that a hierarchy of PARP function may exist under which transcriptional repression may be abrogated in response to DNA damage due to a higher affinity of PARP for DNA breaks and its dissociation from DNA following protein automodification (Fig. 7). This concept integrates PARP functions in DNA repair (a nick-protection mechanism) (15) and in transcriptional control of gene(s) involved in immediate cellular response to ionizing radiation and DNA damaging drugs.

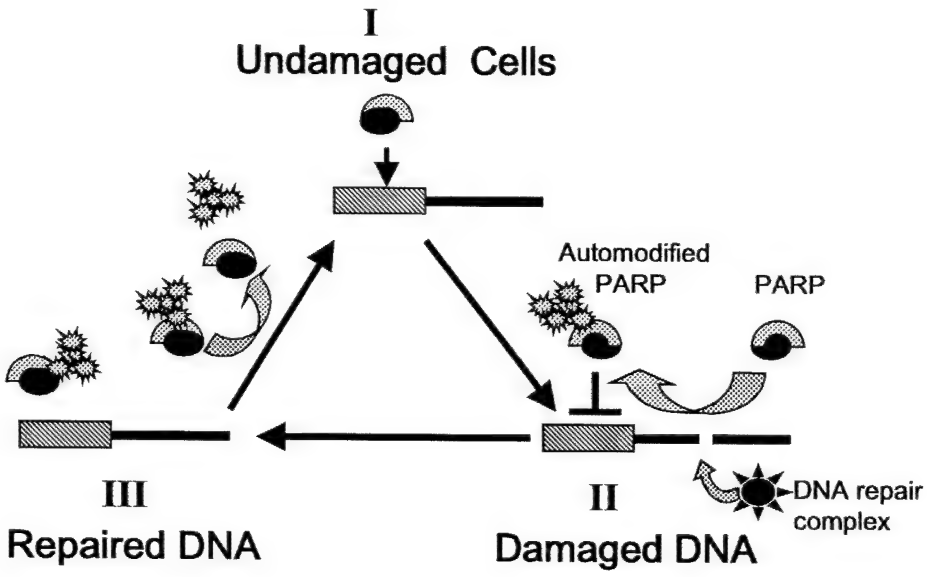


Figure 7. A model for PARP-mediated regulation of transcription. I, in undamaged cells, unmodified PARP molecules bind to the DNA secondary structures within the gene promoter (denoted by a striped box). Such macromolecular interactions between PARP protein and a promoter region constitute a repressor function for PARP in transcription. II, in response to DNA damage, PARP binding to the DNA ends triggers its catalytic activity. Subsequent poly(ADP-ribosy)ation of free and bound PARP in the presence of intracellular NAD^+ prevents its interaction with the promoter regions. This alleviates the PARP-mediated block on the promoter and up-regulate transcription of its own and other genes involved in the DNA damage response. III, the DNA-binding activity of PARP is restored following DNA damage repair and the degradation of the ADP-ribose polymers by poly(ADP-ribose) glycohydrolase leading to re-assembly of PARP-promoter complexes and inhibition of transcription.

KEY RESEARCH ACCOMPLISHMENTS

- 5'-regulatory elements (1.3 kb enhancer and 0.6 kb promoter) of the human PSA gene were isolated and cloned into mammalian expression vector, pcDNA 3.1(-).
- Recombinant plasmid, pCMV-DBD/F, was generated. This construct permits constitutive expression of the human PARP-DBD under control of the CMV promoter.
- Recombinant plasmid, pPSA(EP)-DBD/F, was generated. This construct permits the expression of the human PARP-DBD in androgen-inducible and PSA-dependent fashion.
- LNCaP cell subline stably expressing functionally active PARP-DBD under control of CMV promoter was developed.
- LNCaP cell subline stably expressing PARP-DBD under control of PSA promoter regulatory elements was developed.
- We demonstrated that PSA promoter driven PARP-DBD expression in LNCaP cells shows tissue-specificity and responsiveness to androgens.
- We demonstrated that DNA-binding activity of PARP is essential for its function in regulation of gene expression, and that PARP-DBD may interfere with PARP-mediated regulation of transcription.
- We demonstrated that enforced expression of the PARP-DBD sensitizes LNCaP cells to DNA-damaging treatments

REPORTABLE OUTCOMES

Manuscripts:

1. **Soldatenkov VA,** Smulson M: Poly(ADP-ribose) polymerase in DNA damage response pathway: implications for radiation oncology (REVIEW). *Int. J. Cancer*, 90: 59-67, 2000
2. **Soldatenkov VA**, Chasovskikh S, Potaman VN, Trofimova I, Smulson ME, Dritschilo A: Transcriptional repression by binding of poly(ADP-ribose) polymerase to promoter sequences. *J. Biol. Chem.*, 277: 665-670, 2002
3. **Soldatenkov VA**, Trofimova I, Rouzant A, McDermott F, Dritschilo A, Notario V: EWS-Fli 1 protein downregulates the human poly(ADP-ribose) polymerase promoter and inhibits apoptosis in Ewing's sarcoma cells *Oncogene,* 21: 2890-2895, 2002
4. Newman RE, **Soldatenkov VA**, Dritschilo A, Notario V: Poly(ADP-ribose) polymerase turnover alterations do not contribute to PARP overexpression in Ewing's sarcoma cells *Oncology Reps,* 9: 529-532, 2002
5. Protozanova E, Demidov VV, **Soldatenkov VA**, Chasovskikh S, Maxim D. Frank-Kamenetskii M.D. Tailoring the DNA-processing enzymes activity via the PNA-induced DNA looping. *EMBO Reps.* 3: 956-961, 2002
6. Trofimova I, Dimtchev A, Jung M, Rosenthal D, Smulson M, Dritschilo A, **Soldatenkov VA**: PARP-directed intervention for sensitizing prostate cancer to genotoxic agents. *Cancer Research,* 62: 6879-6883, 2002

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1. Trofimova I, McDermott F, Dritschilo A, Notario V, **Soldatenkov VA.** Down-regulation of ETS1 transcription factor inhibits apoptosis of Ewing's tumor cells. 92nd An Mtg AACR, New Orleans, LA. Proceedings, p. 637, 2001
2. **Soldatenkov VA**, Chasovskikh S, Potaman VN, Dritschilo A. Transcriptional autoregulation of the human poly(ADP-ribose) polymerase gene. 48th An Mtg Radiat. Res. Soc. San Juan, Puerto Rico, p.141-142, 2001
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6. **Soldatenkov VA**, Chasovskikh S, Trofimova I, Potaman VN, Smulson M., and Dritschilo A. Poly(ADP-ribose) polymerase and DNA damage-dependent gene regulation. *Environmental and Molecular Mutagenesis*, 39 (S 33): 60, 2002
7. **Soldatenkov VA.** Gene therapy for prostate cancer: intervention by targeting DNA-damage response. 7th World Congress on Advances in Oncology and 5th International Symposium on Molecular Medicine, Crete, Greece. *International J of Molecular Medicine*, 10 (S1): S8, 2002

Cell lines developed:

- LNCaP/CMV-DBD, LNCaP cells stably transfected with pCMV-DBD/F
- LNCaP/PSA(e/p)-DBD, LNCaP cells stably transfected with pPSA(e/p)-DBD/F

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CONCLUSION

Although many prostate cancer cells are deficient in DNA mismatch repair, they are resistant to ionizing radiation and DNA-damaging drugs. Therefore, targeting molecular components that are critically involved in maintenance of genome stability is a promising approach directed at overcoming intrinsic tumor cell resistance to DNA-damaging treatments. From this point of view, the strategy described here represents a novel starting point for the design of PARP-based molecular therapies targeting prostate cancer *in vivo*. First, this approach utilizes tissue-specific (prostate carcinoma) and treatment-specific (DNA damage) gene therapy for prostate cancer. Next, to avoid the potential side effects due to expression of PSA in tissues other than prostate, tumor cells are targeted using an agent that is not functionally active in the absence of massive DNA damage and, therefore, would not be toxic to cells outside of the irradiated volume or pose a genetic risk to the patient. Furthermore, PARP-DBD mediated cell death is independent of cell proliferation states since both non-dividing cells and rapidly proliferating cancer cells cannot survive the massive accumulation of long-lived damage in the genome. Thus, targeting tumor cells with the PARP-DBD can be beneficial especially for the control of prostate cancer, since prostate cancers usually grow very slow. These properties of the PARP-DBD are in marked contrast to conventional chemotherapeutic drugs, which primarily target proliferating cells.

In summary, the plasmid vector developed in this study permits the expression of the human PARP-DBD in an androgen-inducible and PSA-dependent fashion and sensitizes prostatic adenocarcinoma cells to DNA-damaging treatments. These results provide a proof-of-principle for a novel therapeutic strategy to control prostate cancer.

APPENDIX

1. Reprint of Journal article: *Int. J. Cancer*, 90: 59-67, 2000
2. Reprint of Journal article: *J. Biol. Chem.*, 277: 665-670, 2002
3. Reprint of Journal article: *Cancer Research,* 62: 6879-6883, 2002

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Poly(ADP-ribose) Polymerase in DNA Damage-Response Pathway: *Implications for Radiation Oncology*

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SUMMARY **Poly(ADP-ribose) polymerase (PARP) catalyzes the transfer of successive units of ADP-ribose moiety from NAD^+ covalently to itself and other nuclear acceptor proteins. PARP is a zinc finger-containing protein, allowing the enzyme to bind to either double- or single-strand DNA breaks without any apparent sequence preference. The catalytic activity of PARP is strictly dependent on the presence of strand breaks in DNA and is modulated by the level of automodification. Data from many studies show that PARP is involved in numerous biological functions, all of which are associated with the breaking and rejoining of DNA strands, and plays a pivotal role in DNA damage repair. Recent advances in apoptosis research identified PARP as one of the intracellular "death substrates" and demonstrated the involvement of polymerase in the execution of programmed cell death. This review summarizes the biological effects of PARP function that may have a potential for targeted sensitization of tumor cells to genotoxic agents and radiotherapy.** *Int. J. Cancer (Radiat. Oncol. Invest.) 90, 59–67 (2000).* **© 2000 Wiley-Liss, Inc.**

Key words: poly(ADP-ribose) polymerase; ionizing radiation; DNA damage repair; cell death; gene regulation

INTRODUCTION

Poly(ADP-ribose) polymerase (PARP, EC 2.4.2.30) is a chromatin-associated enzyme that catalyzes the transfer of successive units of ADP-ribose moiety from NAD^+ covalently to itself and other nuclear acceptor proteins. The catalytic activity of PARP is strictly dependent on the presence of strand breaks in DNA and is modulated by the level of automodification. On the basis of the nature and functions of acceptor proteins and the dependency of PARP on DNA strand breaks for catalytic activity, it has been suggested that PARP-dependent protein modification has a role in important cellular processes that require DNA cleavage and rejoining reactions, such as DNA replication, recombination and repair, cell cycle regulation, cell differentiation, and neoplastic transformation [reviewed in 1–5]. Much of the experimental data in support of these functions derive from studies of the effect of chemical inhibitors of polymerase activity [6–8]. Because these chemical inhibitors lack specificity and exert pleiotropic effects not directly related to PARP function, such studies remain controversial [9,10].

Recent advances in molecular biology and genetics of the PARP gene have bridged the gap be-

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tween the proposed roles for the polymerase and the factual molecular basis of its function. In addition to its role in DNA damage repair, the involvement of PARP has been implicated in regulation of gene expression [11–14] and execution of programmed cell death [15–18]. Cumulatively, these findings suggest PARP plays a fundamental role both in normal function of eukaryotic cells and in cellular response to DNA damage. This article reviews the role for PARP in cellular responses to DNA damage and attempts to integrate this knowledge with potential implications of PARP- targeted interventions for sensitizing mammalian tumor cells to radiation therapy and chemotherapy using genotoxic agents.

POLY(ADP-RIBOSYLATION) OF NUCLEAR PROTEINS

The nuclear enzyme PARP is found in almost all eukaryotic cells [1], with the only known exception being yeast [19]. PARP is a major nonhistone chromosomal protein and is present in large concentration (approximately 1 enzyme molecule per 50 nucleosomes) in eukaryotic nuclei [20]. The polymerase has a high binding affinity for blunt ends of DNA and 3′ single-base overhands compared with long overhands; the affinity of PARP for nicks in DNA is fourfold less than for blunt ends [21]. The catalytic activity of polymerase is strongly stimulated after binding of the enzyme to broken DNA ends. Benjamin and Gill [22] have shown a linear relationship between the number of nicks in DNA and polymerase activity. Moreover, the type of break is also significant for PARP stimulation [23]. Yamanaka et al. [20] estimated that only about 1% of the total polymerase molecules would be active under physiological conditions and in the absence of massive production of DNA strand breaks.

This enzyme transfers the ADP-ribosyl part of NAD^+ either to nuclear proteins or to itself to generate long, branched, and negatively charged poly(ADP-ribose) chains. When PARP is hyper(ADP-ribosyl)ated, it acquired a high negative charge, becomes repulsed from DNA, and thus is inactivated [23]. On modified proteins, poly(ADP-ribose) turns over very rapidly, with a half-life of less than 1 min [24]. The ADP-ribose polymer is hydrolyzed by poly(ADP-ribose) glycohydrolase to yield ADP-ribose, and the latter is subsequently hydrolyzed by phosphodiesterase to 5′-AMP and ribose 5-phosphate as final products [25]. Thus, the balanced actions of poly(ADP-ribose) polymerase and glycohydrolase could mediate transient physiological changes in chromatin structure and regulate functional activity of nuclear proteins.

The gene for PARP was cloned [26] and mapped to chromosome 1 at q41-q42 [27]. The cDNA encoding the human enzyme (approximately 3.7 kb length) contains an open reading frame coding for a 1,014 amino acids polypeptide with a calculated molecular weight of 113 kDa [26,27]. Three distinct functional domains are recognized by limited proteolysis of the purified enzyme: 1) a 46 kDa N-terminal domain, 2) a 22 kDa centrally located automodification domain, and 3) a 54 kDa carboxy-terminal catalytic domain [28]. The amino-terminal DNA-binding domain contains two putative zinc-binding motifs that may be responsible for the protein's specificity to bind double and single-strand breaks on DNA [29]. The automodification domain of PARP contains protein–protein binding motifs involved in recognition and stabilization of homodimeric and heterodimeric PARP-DNA complexes [30] and 15 highly conserved Glu residues that may act as automodification sites [31]. The C-terminal region is the NAD^+-binding site [32].

The binding of PARP to the broken DNA ends triggers a 500-fold stimulation of ADP-ribose polymer synthesis [33] and subsequent modification of various nuclear acceptor proteins with very strong polyanion. Poly(ADP-ribosyl)ation of proteins has profound effects on chromosomal architecture and function of chromosome-associated proteins because most of the molecular targets for PARP are DNA-binding proteins. The data summarized in Table 1 [11,12,34–53] indicate that the protein–protein or protein–DNA interactions involving PARP may have biological consequences for 1) metabolism of nucleic acids, 2) modulation of chromatin structure, 3) regulation of gene expression, and 4) maintenance of genome stability.

TRANSCRIPTIONAL REGULATION OF PARP GENE EXPRESSION

The functional involvement of poly(ADP-ribose) in various physiological phenomena such as cell differentiation, cell proliferation, and transformation of eukaryotic cells suggests that the PARP gene is highly regulated at the level of transcription. Indeed, the changes in polymerase expression levels have been demonstrated under various cellular conditions. For instance, Yamanaka et al. [20] estimated that there are 5×10^5 polymerase molecules per cell in resting peripheral blood lymphocytes; this figure increases fourfold after stimulation to proliferation with phytohemagglutinin. Furthermore, changes in levels of PARP mRNA have been shown during cell differentiation [54], cell cycle

Table 1. Protein Substrates for Poly(ADP-ribose) Polymerase

Function	Protein-acceptor	Reference
DNA metabolism	DNA polymerase α	[34]
	DNA polymerase β	[34]
	DNA ligase I	[34]
	DNA ligase II	[34]
	Topoisomerase I	[35]
	Topoisomerase II	[36]
	Ca^{2+}, Mg^{2+}-endonuclease	[37]
	Terminal transferase	[34]
	Poly(ADP-ribose) polymerase	[38]
RNA metabolism	RNA polymerase I	[39]
	RNA polymerase II	[40]
	Ribonuclease	[41]
Protein metabolism	20S Proteasome	[42]
Chromatin structure	Histones	[43]
	HMG proteins	[44]
	LMG protein	[45]
	Lamins	[46]
Gene regulation	Fos	[47]
	p53	[48]
	$TF_{II}C$	[49]
	$TF_{II}F$	[11]
	TEF-1	[12]
Other regulatory proteins	DNA-dependent protein kinase	[50]
	Numatrin/B23	[51]
	Nucleolin/C23	[52]
	PCNA	[53]

progression [55,56], lymphocyte activation [20,57], and liver regeneration [58]. However, despite numerous studies on the function of PARP in mammalian cells and recent advances in the molecular genetics of the PARP encoding gene, very little is known about mechanisms for regulation of PARP gene transcription.

The 5′-regulatory region of the PARP gene has been isolated from normal human liver and lymphoid cells [59–61] and from Ewing's sarcoma cells that express PARP at unusually high levels [62]. This upstream gene promoter exhibits features typical of TATA-deficient, G+C-rich class of promoters. Genes controlled by this type of promoter include many that are highly regulated and functionally important [reviewed in 63]. Several lines of evidence have suggested that PARP gene expression is also regulated at the level of transcription. First, previously recognized features of the PARP promoter have indicated a number of possible trans-acting factors including the presence of dyad symmetry units, SP1, and AP-2 transcription factor binding sites [59,60,64]. Next, the induction of PARP gene expression in response to cAMP and phorbol esters has been demonstrated in vitro and in vivo [60]. More recently, a mechanism of PARP gene autoregulation has been proposed, involving the specific interactions between PARP protein and cruciform structures located in the distal region of the PARP promoter [61].

PARP gene expression is maintained at relatively low levels in most human tissues, suggesting the existence of intrinsic mechanisms for the autoregulation of the endogenous content of PARP protein [54]. In contrast, Ewing sarcoma (EWS) cells accumulate PARP mRNA, protein, and polymerase activity [65] at levels that would cause the death of other cell types. Therefore, EWS cells represent a unique model for investigating PARP transcriptional regulation with regard to the identification of the transcription effectors responsible for the unusually high levels of PARP in these primitive neuroectodermal tumor cells. The 5′-flanking region of the PARP gene from EWS cells has been recently cloned and analyzed [62]. Nucleotide sequence analysis of the cloned fragment revealed no remarkable differences in the sequences reported for PARP promoter regions isolated from normal human cells [59,60]. These data suggest the enhanced levels of PARP in EWS cells relative to normal cells could be due to transcriptional upregulation of the PARP promoter rather than to sequence differences within the PARP 5′-regulatory region. Indeed, it was demonstrated that transcriptional activity of the PARP promoter correlates with protein expression levels in vitro [62,64]. One remarkable feature of the PARP gene promoter is that it contains multiple ETS-binding sties surrounding the transcription start site. The ETS multigene family encodes a class of eukaryotic transcription factors that share a highly conserved DNA-binding sequence, referred to as the ETS domain [reviewed in 66]. Recently, it has been demonstrated that ETS1 transcription factor is capable of transactivating the PARP promoter in vitro and that PARP gene expression can be modulated in cells stably transfected with antisense Ets1 cDNA [62].

Although these data suggest the existence of a variety of regulatory factors for PARP gene expression, no other endogenous PARP transactivators have been identified to date. Additional studies are required to understand the role of transcriptional factors and cis-acting elements in the regulation of the PARP gene expression. These investigations may provide an approach for the manipulation of endogenous PARP levels in human tumor cells and, therefore, for the modulation of their response to ionizing radiation and DNA-damaging drugs to improve the outcome of antitumor therapies.

PARP SIGNALING DOWNSTREAM OF DNA BREAKS

Initial evidence supporting functional involvement of PARP in DNA repair and maintenance of genomic stability has been obtained from studies using PARP competitive inhibitors (i.e., benzamide and its derivatives). Treatment of cells with chemical PARP inhibitors slows DNA repair, increases the activity of sister chromatid exchanges, and considerably increases the cytotoxicity of DNA-damaging treatments [2,4,8,67]. Although these data indicate that PARP may play a pivotal role in DNA damage repair, the limited specificity of PARP chemical inhibitors often raises questions about the validity of the results and interpretation of these studies [9,10]. Cloning the PARP gene [26,27] has allowed circumvention of most of these problems by using genetically engineered models both in vivo and in vitro. Some of these molecular approaches include the depletion of endogenous PARP protein by antisense RNA induction, the use of deletion mutants of PARP, the use of "knockout" mice with disrupted PARP gene, trans-dominant inhibition of PARP activity by over expression of its DNA-binding domain, and expression of the caspase-resistant PARP mutant in mammalian cells [reviewed in 68, 69–72].

Cell culture systems have demonstrated that PARP is involved in numerous biological functions, all of which are associated with breaking and rejoining DNA strands [68]. Eukaryotic cells expressing PARP antisense cDNA have a pronounced lag in initiation of DNA repair, which results in altered chromatin structure and reduced survival after exposure to DNA-damaging agents [73]. It has been hypothesized that PARP cycles between an unmodified form, which blocks DNA strand ends, and a modified form, which is released from DNA, thereby allowing access of repair enzymes [4]. The "PARP cycling" was recently demonstrated in an in vitro DNA repair system using deletion mutants of PARP [74].

Mice lacking PARP as a result of gene disruption exhibit diverse phenotypes. Whereas animals of one strain show epidermal hypertrophy and obesity [75], those of another strain exhibit growth retardation, aberrant apoptosis, and increased sensitivity to DNA-damaging agents [76]. Furthermore, immortalized fibroblasts derived from exon 2 PARP knockout mice (PARP–/–) exhibit mixed ploidy, including a tetraploid cell population, indicative of genomic instability [77]. Comparative genomic hybridization revealed gains in regions of chromosomes 4, 5, and 14, as well as deletion of a region of chromosome 14 (encompassing the Rb tumor-suppressor gene) in both liver tissue and immortalized fibroblasts derived from the PARP–/– animals. Neither the chromosomal gains nor the tetraploid population were apparent in PARP–/– cells that had been stably transfected with PARP cDNA [77], implicating PARP in the maintenance of genomic stability.

The possible involvement of PARP in cell-cycle checkpoint mechanisms after DNA-damaging treatments has long been suggested [55,56,78]. Excessive turnover of poly(ADP-ribose) in response to DNA damage depletes cells of their NAD^+ and at the same time or shortly thereafter, ATP levels drop [67]. This depletion leads to an overall decrease of cell metabolism and slows down the rate of cell proliferation, thereby strengthening the efficiency of DNA damage repair [79]. However, this effect is not simply the result of a generalized decrease in intracellular ATP levels, but likely to be caused by impaired function of cell-cycle regulatory proteins. Recently, Masutani et al. [80] demonstrated in vitro that PARP can directly block the cell cycle under DNA-damaging conditions by inhibition of cdk activity on pRB-phosphorylation. Furthermore, a functional association of PARP and tumor-suppressor protein p53 has recently been demonstrated. It was shown that p53 is poly(ADP-ribosyl)ated in vitro by purified PARP [81], and that PARP is required for rapid accumulation of p53, activation of p53 sequence-specific DNA binding, and its transcriptional activity after DNA damage [82]. In turn, the accumulation of p53 leads to inhibition of cell-cycle progression, thereby preventing the proliferation of damaged cells [83].

Taken together, these data suggest that PARP is an important element of cellular response to genotoxic stress acting as a component of the DNA-repair machinery and as part of the checkpoint pathway, thereby preventing cells carrying damaged DNA from unrestrained DNA replication or entering mitosis (Fig. 1). Therefore, inactivation of PARP may have therapeutic implications, because it will render cell particularly sensitive to DNA damaging agents due to impairment of cellular recovery from DNA damage.

PARP AND PROGRAMMED CELL DEATH

The "cytoprotective" function of PARP is dramatically changed when the massive DNA damages cannot be effectively repaired. Damaged cells that fail to pass the DNA damage checkpoint are elimi-

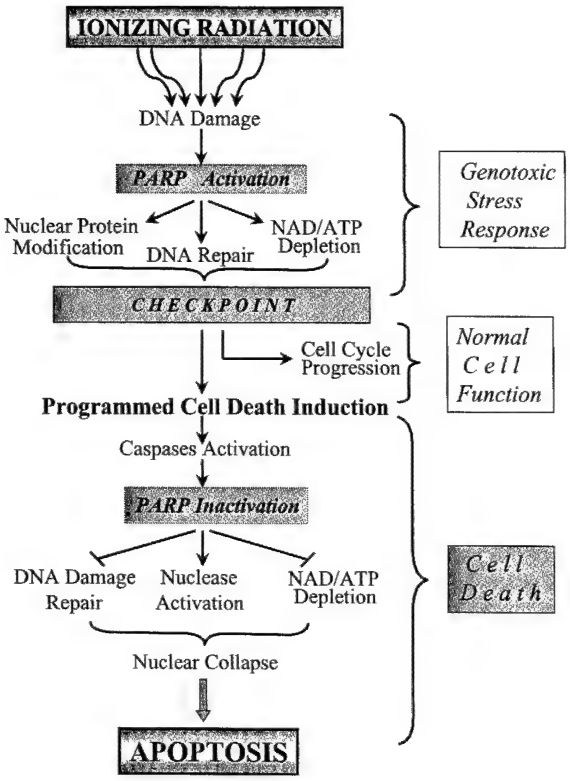

Fig. 1. Poly(ADP-ribosyl)ation of nuclear proteins in cellular response to DNA damage.

nated by a programmed self-destruction process commonly termed apoptosis [84]. Upon activation of cellular suicide (apoptosis), PARP is recruited to participate in the execution of the cell death program, serving as a "death substrate."

The requirement of PARP for execution of apoptotic pathways has been recently demonstrated by using immortalized fibroblasts derived from wild-type (PARP+/+) and PARP knockout (PARP–/–) mice [85]. Whereas immortalized PARP+/+ cells showed the early burst of poly(ADP-ribosyl)ation and rapid apoptotic response to anti-Fas treatment, PARP–/– fibroblasts exhibited neither the early poly(ADP-ribosyl)ation nor any of the biochemical or morphological changes characteristic of apoptosis when similar treated. Stable transfection of PARP–/– fibroblasts with wild-type PARP rendered the cells sensitive to Fas-mediated apoptosis. These results suggest that PARP and poly(ADP-ribosyl)ation may trigger key steps in the apoptotic program.

It has been well recognized that limited proteolysis of PARP by caspases family of cysteine proteases is an early event or perhaps a prerequisite for the execution of programmed cell death in various mammalian cells [15–17,86]. The caspase-specific DEVD motif resides adjacent to the nuclear localization signal of PARP protein. Cleavage of PARP at this site results in the separation of the two zinc-finger DNA-binding motifs in the amino terminus of PARP from the automodification and catalytic domains located in the carboxyl terminus of the enzyme [17]. Consequently, this cleavage excludes the catalytic domain from being recruited to the sites of DNA fragmentation during apoptosis and presumably disables PARP from coordinating subsequent repair of genome maintenance events [74]. Recently, the irreversible finding of the 24 kDa proteolytic fragment of PARP to broken DNA ends has been directly demonstrated by atomic force microscopy [87]. The significance of PARP cleavage and DNA-binding domain (DBD) of PARP (PARP-DBD) accumulation for execution of apoptosis has been investigated by using stable cell lines constitutively expressing PARP-DBD [18,70]. Enforced expression of the N-terminal fragment of PARP containing the DBD in cultured mammalian cells led to trans-dominant inhibition of the resident PARP activity and delay in DNA strand break rejoining. Furthermore, exposure of PARP-DBD–expressing cells to DNA damaging agents and ionizing radiation resulted in a marked reduction of cell survival, increased frequency of sister chromatid exchanges, inhibition of cell proliferation, and induction of apoptosis [18,70].

PARP cleavage by caspase(s) occurs early in apoptosis, before or soon after the appearance of internucleosomal fragmentation of DNA [15–17], a biochemical hallmark for programmed cell death. Although several nucleases are implicated in the mechanisms of chromosomal DNA disintegration in dying cells [reviewed in 88], it has been suggested that Ca^{2+}/Mg^{2+}-dependent endonuclease (CME) is responsible for cleavage of genome DNA at internucleosomal sites [89] during the late phase of apoptosis execution in most of the eukaryotic cells. This endonuclease is maintained in a latent form by poly(ADP-ribosyl)ation [37]. Consequently, inactivation of PARP by caspases may result in CME derepression and thereby promote fragmentation of genome DNA. The plausibility of such a mechanism has been demonstrated in vitro using endonucleolysis of isolated nuclei as a model in the presence of PARP inhibitors [90]. In addition, the inactivation of poly(ADP-ribosyl)ation might facilitate the accessibility of endonucleases to chromatin in dying cells. Indeed, downregulation of PARP expression by antisense mRNA delivery to cells resulted in an increased accessibility of micrococcal nuclease to nuclear DNA in chromatin [73].

Recent studies suggest that apoptosis is an energy-requiring process and that an intracellular adenosine triphosphate level influences the mode of cell death—apoptosis or necrosis [91]. Rendering PARP catalytically inactive by caspase cleavage would prevent the decrease in the content of NAD^+ and ATP, thus providing the source of intracellular energy needed for execution of the cell death program. This idea has been supported in recent studies designed to prevent PARP proteolysis by introduction of point mutations into the DEVD cleavage site to produce the "uncleavable" mutant protein. The mammalian cells expressing the caspase-resistant PARP protein in a PARP-null background exhibited accelerated tumor necrosis factor-alpha–induced cell death and increased apoptosis [92,93]. These data suggest that PARP cleavage prevents necrosis associated with depletion of NAD^+ and ATP to ensure appropriate execution of programmed cell death. However, the PARP-mediated changes in intracellular NAD^+ and ATP content do not always occur in cells undergoing apoptosis [94,95]. Therefore, the cause-effect relationship of NAD^+ depletion to apoptosis execution should be viewed critically.

CONCLUDING REMARKS

Recent developments in molecular genetics of the PARP gene and availability of PARP-deficient cells from transgenic knockout mice allowed reevaluation of the biological functions of this unique modification of nuclear proteins in the maintenance of cell surveillance. An early transient burst of poly(ADP-ribosyl)ation in response to DNA damage and subsequent inactivation of PARP during an execution stage of apoptosis indicate that PARP has active and complex roles in mechanisms of cellular stress response and in pathways leading to programmed cell death. PARP activity appears to be necessary for maintenance of genome stability in normal living cells and during the adaptive phase of cellular response to the genotoxic stress. This "pro-life" function of PARP is switched to a "pro-death" function, when cells are not capable of enduring the sustained DNA damage in the genome and are to be eliminated via apoptosis. The cleavage of PARP that occurs during the execution phase of apoptosis might help avoid unnecessary DNA repair in dying cells, facilitate nuclear disintegration, and preserve the energy needed for the biochemical cascade of events culminating in apoptosis, thus ensuring the completion and irreversibility of the cell death process (Fig. 1). Therefore, the development of gene-engineered approaches to target-specific inactivation of PARP in mammalian cells may lower the apoptosis threshold in cancer cells, thereby enhancing the effectiveness of both chemotherapy agents and radiotherapy. This may lay the groundwork for the long-awaited translation of scientific gains from investigations on PARP function to in vivo treatment of cancer.

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Transcriptional Repression by Binding of Poly(ADP-ribose) Polymerase to Promoter Sequences*

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Poly(ADP-ribose) polymerase (PARP) is a DNA-binding enzyme that plays roles in response to DNA damage, apoptosis, and genetic stability. Recent evidence has implicated PARP in transcription of eukaryotic genes. However, the existing paradigm tying PARP function to the presence of DNA strand breaks does not provide a mechanism by which it may be recruited to gene-regulating domains in the absence of DNA damage. Here we report that PARP can bind to the DNA secondary structures (hairpins) in heteroduplex DNA in a DNA end-independent fashion and that automodification of PARP in the presence of NAD^+ inhibited its hairpin binding activity. Atomic force microscopic images show that *in vitro* PARP protein has a preference for the promoter region of the PARP gene in superhelical DNA where the dyad symmetry elements likely form hairpins according to DNase probing. Using a chromatin cross-linking and immunoprecipitation assay we show that PARP protein binds to the chromosomal PARP promoter *in vivo*. Reporter gene assays have revealed that the transcriptional activity of the PARP promoter is 4–5-fold greater in PARP knockout cells than in wild type fibroblasts. Reintroduction of vectors expressing full-length PARP protein or its truncated mutant (DNA-binding domain retained but lacking catalytic activity) into $PARP^{-/-}$ cells has conferred transcriptional down-regulation of the PARP gene promoter. These data provide support for PARP protein as a potent regulator of transcription including down-regulation of its own promoter.

Poly(ADP-ribose) polymerase (PARP,[1] EC 2.4.2.30) is a chromatin-associated enzyme that catalyzes the transfer of successive units of the ADP-ribose moiety from NAD^+ to itself and other nuclear acceptor proteins (1). PARP is a zinc finger-containing protein, which allows enzyme binding to either double or single strand DNA breaks without any apparent sequence preference (2, 3). The catalytic activity of PARP is strictly dependent on the presence of strand breaks in DNA and is modulated by the level of automodification (4, 5). Data from many studies show that PARP is involved in numerous biological functions, all of which are associated with breaking and rejoining DNA strands, and it plays a pivotal role in DNA damage repair (2, 6–8).

Recent studies have implicated PARP in transcription of eukaryotic genes (9–16). PARP-dependent gene regulation involves poly(ADP-ribosyl)ation of transcription factors, which, in turn, prevents their binding to specific promoter sequences (10). The basal transcription factors TFIIF and TEF-1 as well as transcription factors TATA box-binding protein, YY1, SP-1, cAMP-response element-binding protein, p53, and NFκB are all highly specific substrates for poly(ADP-ribosyl)ation (10, 11, 14, 16). PARP may also interact directly with gene promoters. For instance, recombinant full-length PARP bound the DNA sequences within the MCAT1 regulatory element (11) and to the DF4 protein binding site of the *Pax-6* gene neuroretina-specific enhancer (17). Furthermore, PARP involvement in the active transcriptional DNA-protein complex formation on *Reg* promoter has been recently reported (12). Together these observations suggest that PARP may exert its function in transcription through direct binding to the gene-regulating sequences and through modification of transcription factors by poly(ADP-ribosyl)ation. However, total dependence of PARP function on DNA strand breaks (5) does not provide a mechanism by which it may ADP-ribosylate transcription regulators and be recruited to gene-regulating sequences in the absence of DNA damage.

Based on the ability of PARP to interact with partially unwound DNA (18, 19), we reasoned that DNA secondary structures with single-stranded character may provide potential binding sites for PARP in gene-regulating sequences in the absence of DNA strand breaks. In this work we investigated the interactions between PARP protein and DNA structures of different complexity such as DNA heteroduplexes carrying stable secondary structures and superhelical DNA containing PARP promoter sequences. We found that PARP can recognize noncanonical conformations (hairpins) in a DNA end-independent fashion, and it is capable of *in vitro* binding to the PARP promoter sequences where the dyad symmetry elements may form the cruciforms. Using a chromatin cross-linking and immunoprecipitation assay we show that the human PARP promoter is an *in vivo* target for PARP protein. Further, we show that PARP protein down-regulates its gene promoter and that DNA binding activity of PARP is essential for its function in transcription.

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[1] The abbreviations used are: PARP, poly(ADP-ribose) polymerase; PARP-DBD, DNA-binding domain of PARP; AFM, atomic force microscopy; nt, nucleotide(s).

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The plasmid pPR-PARP was constructed by cloning the 5′-flanking region of the human PARP gene (from −899 to +156) fused to a chloramphenicol acetyltransferase reporter (20) into pcDNA 3.1 (Invitrogen) modified to remove the cytomegalovirus promoter. The 5′-deletion mutant of the PARP promoter (pΔPR-PARP) was generated as described previously (20). The expression plasmid pCD12 containing cDNA for human PARP has been described previously (21). pPARP-DBD was constructed by cloning the PCR-generated fragment of cDNA (22) for human PARP-DBD (amino acids 1–303) tagged at its carboxyl terminus with a sequence encoding four FLAG epitope tags into pcDNA 3.1. The integrity of all constructs was confirmed by sequence analysis.

DNA Heteroduplex Formation and Isolation—Heteroduplex formation between 301-bp *Pvu*II-*Pvu*II fragments of pUC8 and a similar fragment of pUC8F14C and isolation of the heteroduplex isomers were performed as described previously (23). Briefly, 10 μl of hybridization mixture containing 1 pmol of each DNA fragment in 100 mM NaCl, 50 mM Tris-HCl, pH 7.9, 1 mM dithiothreitol, 10 mM $MgCl_2$ were incubated stepwise at 100 °C (1 min), 85 °C (10 min), and 70 °C (60 min) and then cooled to room temperature. Hybridization products were run in a 5% native polyacrylamide gel in 90 mM Tris borate (pH 8.3), 2.5 mM EDTA, and bands of heteroduplex fragments, which migrate slower than correctly annealed parental fragments (23), were excised. After an additional purification step using an UltraClean 15 DNA purification kit (MoBio, Solana Beach, CA), isolated heteroduplexes were resuspended in 60 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.8), and aliquots were taken for strand identification by sequencing and atomic force microscopy (AFM) analysis.

Supercoiled Topoisomer Preparation—Each of eight fractions of differently supercoiled DNA (topoisomers) was prepared by incubating 5 μg of plasmid DNA purified by CsCl density gradient with 20 μl of topoisomerase I-containing nuclear extract from HeLa cells (24) in the presence of appropriate concentrations of ethidium bromide (0–13 μM) in 200 μl of reaction buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.6) (25). Average superhelical densities of resultant topoisomer fractions were calculated as $\sigma = 10.5\tau/N$, where N is the number of base pairs in the plasmid, and τ is the number of superhelical turns determined by the band counting method after topoisomer separation in an agarose gel in the presence of chloroquine (26).

Assay for Base-unpaired Sites—The sequence of the 1.1-kb insert was analyzed for potential hairpin formation using MFOLD software.[2] The free energies of potential hairpins were calculated for single-stranded DNA at 37 °C in a solution containing 150 mM monovalent cation and 1 mM Mg^{2+}. To detect unwound regions in supercoiled DNA, 1 μg of each topoisomer prepared in a reaction with topoisomerase I was incubated on ice with 0.5 units of nuclease P1 (Invitrogen) in 10 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 50 mM NaCl at 37 °C for 10 min. The reaction was terminated by phenol/chloroform extraction, and DNA was recovered by ethanol precipitation. Following the *Eco*RI digestion to release a promoter-containing 1.1-kb insert, DNA was 3′-end-labeled using [α-^{32}P]dATP and the Klenow fragment of DNA polymerase from *Escherichia coli* (New England Biolabs). The resultant products were separated in their single-stranded forms on a 1.5% alkaline agarose gel in 50 mM NaOH (pH 12.5), 1 mM EDTA.

PARP Binding Reactions—A recombinant full-length human PARP (R&D Systems) was used in DNA binding reactions at a 4:1 molar ratio (protein to DNA) under the ionic conditions required for optimal PARP activity (4, 21). The heteroduplex DNA (23) containing stable 50-bp hairpin arms was used in PARP binding reactions. Parental duplexes (fragments of pUC8 and pUC8F14C plasmids) were used as controls in these experiments. For PARP binding reactions with the supercoiled or topologically relaxed DNA, plasmids were predigested with exonuclease III to exclude the presence of nicks in the DNA template (19). To analyze the interactions of PARP protein with the promoter region in supercoiled plasmids, bound PARP was cross-linked to DNA with 0.5% glutaraldehyde for 2 min at 37 °C, and the 1.1-kb *Eco*RI-*Eco*RI fragment containing the PARP promoter region was isolated and purified on Sephadex G25 spin columns equilibrated with the deposition buffer (10 mM HEPES, pH 7.3, 1 mM $MgCl_2$).

Chromatin Cross-linking and Immunoprecipitation—Ewing's sarcoma cells A4573 (kindly provided by Dr. T. Kinsella, University of Wisconsin, Madison) were grown and maintained in Eagle's minimal essential medium (Invitrogen). Formaldehyde (Fisher) was added directly to the cell culture medium to a final concentration of 1%, and fixation proceeded at 37 °C for 10 min as described in the ChIP assay protocol (Upstate Biotechnology). Immunoprecipitation was performed with rabbit polyclonal anti-PARP antibody (Cell Signaling Technology). Cross-links were reversed by heating to 65 °C for 4 h in the presence of 200 mM NaCl followed by PCR analysis of DNA for the detection of the PARP promoter sequences using upstream (5′-TGTCA ACCCA GAGAT GGCAT-3′) and downstream (5′-AACTA CTCGG GAGGC TGAA-3′) PCR primers designed according to the reported sequence data for the PARP 5′-region of the human PARP gene (27). Immunocapture of PARP from cross-linked chromatin was analyzed by immunoblotting with goat polyclonal anti-PARP antibody (1:1000, R&D Systems) as described previously (20).

Sample Preparation and Imaging with AFM—DNA samples or PARP-DNA binding reaction product in Mg^{2+}-containing buffer (28) were deposited on an anatomically flat mica surface, allowed to adsorb for 1 min, rinsed with deionized water, and dried in a gentle nitrogen flow. The AFM images were obtained using a NanoScope IIIa instrument equipped with E-scanner (Digital Instruments, Santa Barbara, CA) operating in a tapping mode in air as described previously (28). The tapping frequency of the 125-μm silicon cantilever was 300–400 KHz, and the nominal scanning rate was set at 1–2 Hz. No less than 150 uncomplexed DNA molecules and 100 PARP-DNA complexes were analyzed in each experiment.

Transfections and Reporter Assays—Mouse embryonic fibroblasts derived from both wild type and PARP knockout mice (29) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). DNA transfections were carried out using a SuperFect reagent (Qiagen) according to the protocol of the manufacturer. The total amount of DNA transfected was held constant with the pcDNA 3.1 (Invitrogen) empty vector. Chloramphenicol acetyltransferase reporter assays were performed as described previously (20) and normalized for transfection efficiency using a co-transfected pSV-β-gal vector (Promega) as an internal control. Each experiment was repeated at least three times, in duplicate, with independent plasmid preparations to assess reproducibility.

RESULTS AND DISSCUSSION

PARP Binds to Hairpins in DNA Heteroduplexes—To investigate the interactions of PARP with DNA, we used AFM, which allows direct visualization of protein and DNA molecules at nanometer resolution (30–32). This approach was preferred to biochemical assays to address the hypothesis that PARP binding to DNA sites other than strand breaks was directed to single strand regions as observed in unwound structures in double-stranded DNA. Alternative DNA secondary structures are not thermodynamically stable in linear DNA fragments and, therefore, are not amenable to investigations of their functional transactions such as protein binding. Accordingly, our experimental approach used model heteroduplex constructs carrying stable DNA secondary structures. We used three-way junction heteroduplexes that contain 106-bp inverted repeats in one DNA strand (23) to form hairpin-like DNA structures (Fig. 1*A*). A representative AFM image shows that heteroduplex molecules have extrusions of the size expected for the 50-bp hairpin in the B conformation and bends at the junction (Fig. 1*B*).

After allowing full-length PARP protein to bind to the model hairpin-containing DNA, AFM images revealed a high incidence of DNA-protein complexes (~60% of all DNA molecules) that were divided into two types based on their locations in the heteroduplexes. In complexes of the first type, PARP associated primarily with DNA ends and less frequently dimerized heteroduplexes end-to-end (Fig. 1*D*) consistent with our previous observations that PARP can link DNA fragments into chain-like structures (28). The most striking observation was the occurrence of the second type, internal DNA-protein complexes (Fig. 1, *C*, *D*, and *E*). Proteins in these complexes resided at the junction site and were not observed in other internal regions of the long arms of the model DNA. Moreover, no internal PARP-DNA complexes were formed with control DNA duplexes

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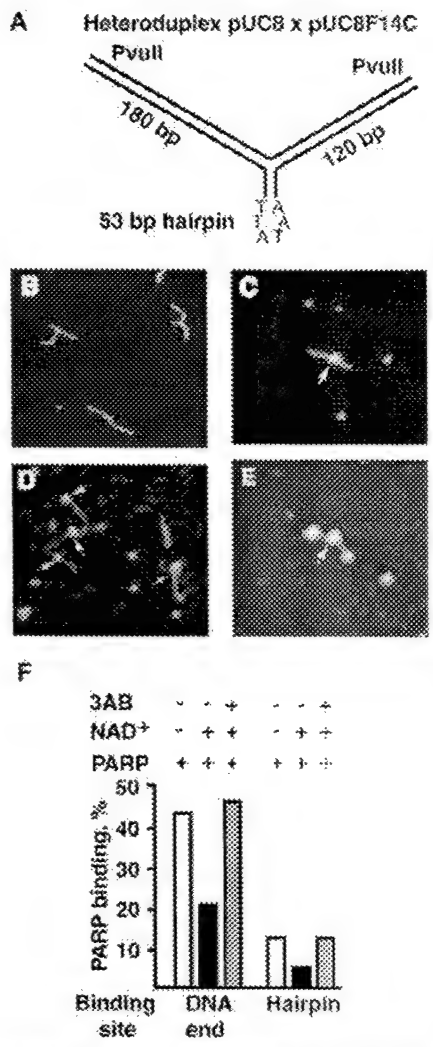

FIG. 1. **Binding of recombinant PARP to three-way DNA junctions.** *A*, schematic representation of heteroduplex DNA with an unpaired region at the apex of hairpin. *B*, AFM images of three-way DNA junctions containing a 50-bp hairpin (visible as the protrusion from the bend near the center of the molecule). *C–E*, representative AFM images of PARP-DNA complexes. End-bound (*yellow arrows*) and internally bound (*white arrows*) PARP molecules are indicated. Images show a 400- × 400-nm surface area. The color scale ranges from 0.0 to 4.0 nm (from dark to bright). *F*, the effects of NAD^+ (0.1 mM) and 3-aminobenzamide (*3AB*) (1 mM) on the interaction of PARP with DNA ends and hairpins. PARP binding to DNA was calculated as the percentage of occurrence of the PARP-DNA complexes to the total number of heteroduplexes scored. Only unobstructed protein-DNA complexes were quantified. The total numbers of DNAs counted in each experiment ranged from 420 to 540 molecules.

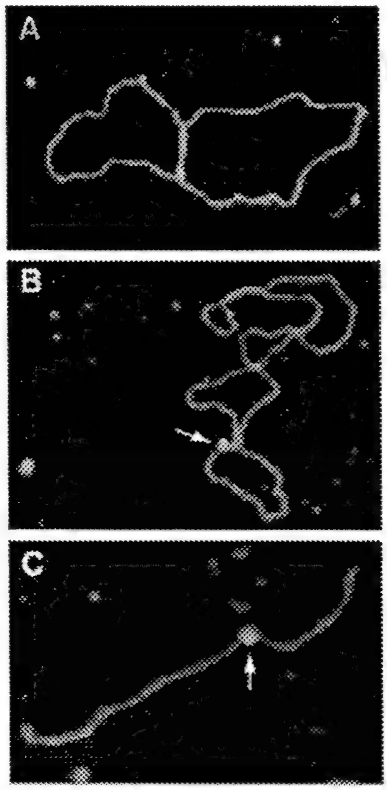

FIG. 2. **Interaction of PARP protein with the 1.1-kb 5′-region of the PARP gene.** *A*, binding of PARP to topologically relaxed pPR-PARP plasmid containing the PARP promoter region (from −899 to +156). *B*, binding of PARP to negatively supercoiled ($\sigma = -0.050$) pPR-PARP plasmid. *C*, AFM images of the PARP protein-promoter complexes. Bound PARP molecules were cross-linked to plasmid DNA with a superhelical density, σ of −0.050, and the promoter-containing fragment (1.1 kb) was isolated for AFM examination. Representative images *A* and *B* show a 700- × 700-nm surface area, and image *C* shows an enlarged surface area (340 × 183 nm). *Arrows* (*B* and *C*) point to the PARP-DNA complex.

(301-bp fragment of pUC8 and 401-bp fragment of pUC8F14C), thus indicating the specificity of PARP binding to hairpin-containing regions in double-stranded DNA. This finding presents a challenge to the generally accepted view that PARP binds only to strand breaks in DNA.

In the presence of NAD^+, PARP bound to DNA strand breaks undergoes auto(ADP-ribosyl)ation, acquiring a high negative charge. Due to the charge repulsion the protein rapidly dissociates from DNA (4, 33, 34). Therefore, we next tested the ability of PARP to bind hairpin-containing DNA under conditions conducive to PARP automodification. Similar to our previous observations of PARP binding to DNA ends (28), NAD^+ significantly decreased PARP affinity to the hairpins. Reversal of this effect was observed in the presence of 3-aminobenzamide (Fig. 1*F*), a potent inhibitor of PARP catalytic activity. The relatively low yield of hairpin-protein complexes suggests that PARP has higher affinity to DNA ends than to hairpins in DNA fragments. These observations indicate that (i) PARP is capable of binding to certain secondary structures (*e.g.* hairpin-containing regions) in double-stranded DNA independently of the presence of DNA ends and (ii) NAD^+-dependent automodification of PARP results in inhibition of its hairpin binding activity.

PARP Protein Binds to the 5′-Flanking Region of the PARP Gene—Accumulating evidence supports the involvement of DNA secondary structures such as hairpins and cruciforms in transcription (34–38). We reasoned that PARP affinity for stem-loops in DNA might influence regulation of transcription in undamaged cells by binding to such domains in promoter regions. To test this hypothesis, we investigated interaction of the PARP protein with the 5′-flanking region of the PARP gene (20). Structurally, the PARP gene promoter is TATA-deficient and G + C-rich, typical of promoters that contain dyad symmetry elements with high propensity to form secondary structures such as cruciforms (39). Secondary structures are favored when DNA is negatively supercoiled and are not thermodynamically stable in linear DNA fragments (40). Therefore, we examined the PARP interactions with supercoiled ($\sigma = -0.050$) and topologically relaxed ($\sigma = 0$) pPR-PARP plasmids (Fig. 2, *A* and *B*). PARP binding reactions were performed using the same DNA to protein molar ratio (4:1) as in experiments with

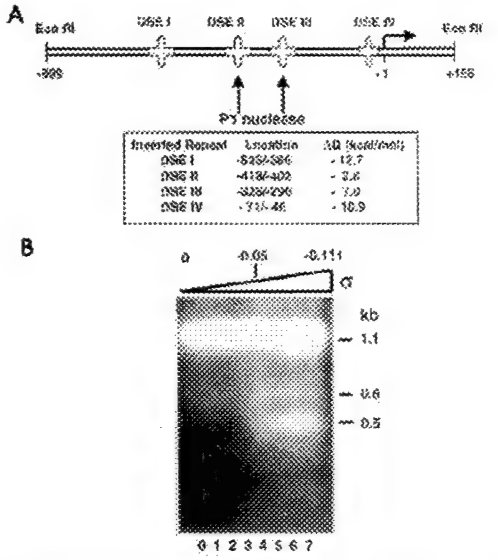

FIG. 3. **Detection of P1 nuclease-sensitive sites in the PARP promoter.** *A*, schematic representation of the human PARP promoter (from −899 to +1). The position of dyad symmetry elements (*DSE*) in the promoter sequence and the hairpin free energies calculated by the MFOLD program are indicated in the *boxed area*. Putative P1 nuclease-sensitive sites are shown with *arrows*. *B*, pPR-PARP topoisomers with superhelical density (σ) ranging from 0 to −0.111 were treated with P1 nuclease. The promoter-containing fragment (1.1 kb) was isolated and analyzed by alkaline agarose gel electrophoresis. The products of P1 nuclease digestion are denoted on the *right*. Topoisomer *fractions 0–7* numbered at the *bottom* had the average σ of 0, −0.019, −0.031, −0.050, −0.065, −0.080, −0.094, and −0.111, respectively.

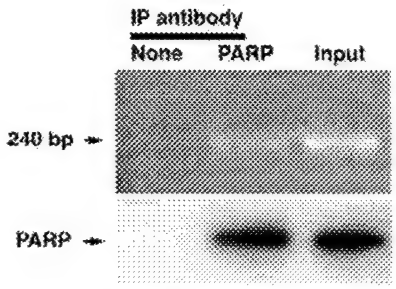

FIG. 4. **PARP protein binds to the 5′-flanking region of the human PARP gene *in vivo*.** Formaldehyde-cross-linked chromatin from asynchronously growing Ewing's sarcoma cells (cell line A4573) was immunoprecipitated using anti-PARP polyclonal antibody. A no-antibody immunoprecipitation was performed for a negative control (*None*). The input sample contains total chromatin before selection by immunoprecipitation. *Top panel*, immunoprecipitated DNA was analyzed by PCR using primers specific for the human PARP promoter. A 240-bp PCR fragment amplified from the PARP promoter sequence is shown. *Bottom panel*, immunoblotting analysis of PARP protein in cross-linked chromatin. *IP*, immunoprecipitation.

hairpin-containing DNA heteroduplexes. AFM imaging of DNA-protein interactions revealed that PARP is capable of binding to supercoiled plasmid in a DNA end-independent fashion. Further, a quantitative evaluation of the AFM images revealed a 3–4-fold higher yield of DNA-protein complexes on a supercoiled plasmid compared with topologically relaxed DNA. These data suggest that the preferential binding of PARP to supercoiled plasmid is attributable to the formation of recognition sites for PARP in torsionally stressed DNA.

To examine PARP protein-promoter interactions *in vitro*, bound proteins were cross-linked to superhelical plasmid (σ = −0.050) with 0.5% glutaraldehyde, and the 1.1-kb fragment containing the promoter region was isolated and examined by AFM. An average of 1.2 protein molecules were bound to the promoter-containing DNA duplex, indicating that PARP recognizes certain relatively infrequent sites in the promoter region (Fig. 2*C*). Although the PARP binding site(s) in its own promoter is yet to be identified, our data might conceivably reflect polymerase interaction with the regions of single-stranded character that can be formed in superhelical DNA. One potential option is the formation of cruciform-like structures since several imperfect inverted repeats have been identified in the promoter sequence by the computer algorithm MFOLD (Fig. 3*A*). In support of this, we observed the appearance of yet unidentified sites in the promoter region that are recognized by the single strand-specific nuclease P1. These sites are generated by unwinding torsional stress in supercoiled DNA with a threshold value of superhelical density σ = −0.050 (Fig. 3*B*) and were not detected in relaxed covalently closed plasmid DNA. Based on the size of P1 nuclease-generated fragments, the positions of the putative unwound sites correspond to imperfect inverted repeat (nt −325/−290) or an AT-rich region with dyad symmetry (nt −418/−403) in the PARP promoter sequences. Although these data suggest that the 5′-flanking region of the PARP gene has the ability to adopt unwound or alternatively base-paired structures, further studies are required to assess functional transactions between PARP protein and such structures and to map PARP binding sites on the promoter.

To analyze the PARP protein-DNA interactions at the human PARP promoter *in vivo* we performed formaldehyde cross-linking and immunoprecipitation experiments. This approach permits analysis of DNA-binding proteins in eukaryotic cells under physiological conditions (41, 42). We observed that anti-PARP antibody effectively immunoprecipitated endogenous PARP protein and the 5′-flanking region of the PARP gene promoter (Fig. 4) from Ewing's sarcoma cells that constitutively express PARP protein (20). This observation indicates that PARP protein is recruited to the human PARP promoter sequences *in vivo*. It remains to be determined whether PARP protein binds to the promoter sequences as a monomer or forms a heterodimer with yet to be identified transcriptional regulator(s). In support of the latter possibility, the physical association of PARP with transcription factors TEF-1, B-MYB, and AP-2 and its involvement in the active transcriptional DNA-protein complex on *Reg* and *Pax-6* promoters have been recently demonstrated (11, 12, 17, 43, 44).

Transcriptional Autoregulation of the Human PARP Gene—The functional significance of PARP interactions with its gene promoter was evaluated by transient transfection assays using immortal fibroblasts ($PARP^{-/-}$) derived from PARP knockout mice (29). We found that the transcriptional activity of the PARP promoter was 4–5-fold greater in $PARP^{-/-}$ cells than in wild type ($PARP^{+/+}$) fibroblasts (Fig. 5*A*). Introduction of plasmid pCD12 carrying PARP cDNA into $PARP^{-/-}$ cells conferred transcriptional down-regulation of the PARP gene promoter (Fig. 5*B*). These data are in accord with the previously reported observations that inducible PARP expression in PARP-producing cells also inhibited PARP promoter activity (45), thus suggesting intrinsic autoregulation of PARP expression. Next we observed that deletion of the −899 to −95 region from the PARP promoter sequences alleviated PARP-mediated transcriptional inhibition (Fig. 5*C*) thus indicating that at least some of the functional sites that are required for PARP-mediated down-regulation of transcription may reside upstream of the minimal PARP promoter (nt from −95 to +156). This suggestion agrees with our earlier observations that the PARP promoter region (nt −420/−290), harboring two putative unwound sites (at nt −418/−403 and −325/−290) (Fig. 3), is involved in negative control of the PARP promoter in cells

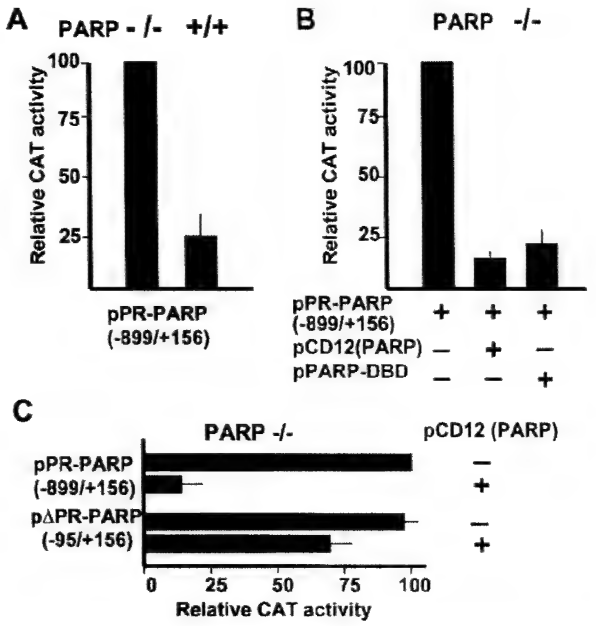

FIG. 5. **PARP protein is a transcriptional repressor.** *A*, PARP promoter transcriptional activity in wild type ($PARP^{+/+}$) and $PARP^{-/-}$ fibroblasts. *B*, expression of human PARP or its DNA-binding domain down-regulates promoter activity. $PARP^{-/-}$ cells were co-transfected with pPR-PARP and plasmids encoding for full-length PARP (pCD12) or its truncated mutant (*pPARP-DBD*). *C*, deletion of the distal region (−899 to −95) alleviates transcriptional repression by PARP protein. Vectors containing the PARP promoter (*pPR-PARP*) or its 5′-deletion mutant (*pΔPR-PARP*) were transiently co-transfected with the PARP-expressing vector into $PARP^{-/-}$ fibroblasts. Chloramphenicol acetyltransferase (*CAT*) activity of pPR-PARP in $PARP^{-/-}$ cells was arbitrarily taken as 100%. Means of triplicate experiments normalized by co-transfected β-galactosidase and S.D. are indicated.

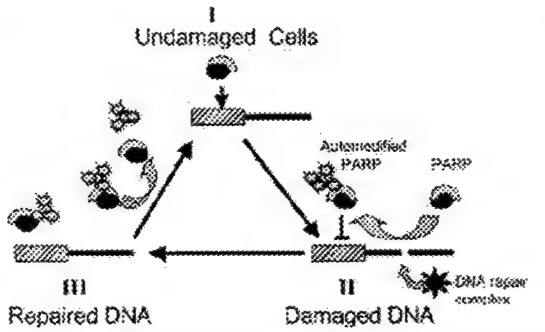

FIG. 6. **A model for PARP-mediated regulation of transcription.** *I*, in undamaged cells, unmodified PARP molecules bind to the DNA secondary structures within the gene promoter (denoted by a *striped box*). Such macromolecular interactions between PARP protein and a promoter region constitute a repressor function for PARP in transcription. *II*, in response to DNA damage, PARP binding to the DNA ends triggers its catalytic activity. Subsequent poly(ADP-ribosyl)ation of free and bound PARP in the presence of intracellular NAD^+ prevents its interaction with the promoter regions. This alleviates the PARP-mediated block on the promoter and up-regulates transcription of its own and other genes involved in the DNA damage response. *III*, the DNA binding activity of PARP is restored following DNA damage repair and the degradation of the ADP-ribose polymers by poly(ADP-ribose) glycohydrolase leading to reassembly of PARP-promoter complexes and inhibition of transcription.

naturally overexpressing PARP protein (20). To address the question whether catalytic activity of PARP is required for transcriptional down-regulation, the amino-terminal fragment of human PARP (amino acids 1–303) encompassing the region that encodes two zinc fingers of the enzyme and the proximal (amino acids 200–220) helix-turn-helix motif (22) was transiently expressed in $PARP^{-/-}$ cells. Co-transfection of the reporter gene (pPR-PARP) and a vector (pPARP-DBD) expressing a truncated PARP mutant (that contains the DNA-binding domain but lacks catalytic activity) resulted in transcriptional down-regulation of the PARP promoter in cells with a PARP-negative background (Fig. 5*B*), thus indicating that PARP-mediated inhibition of transcription was independent of PARP catalytic activity. Together these data demonstrate that PARP protein is a potent repressor of transcription when targeted to promoter and that its DNA binding activity is necessary and sufficient for transcriptional repression. However, we cannot rule out the possibility of cooperative interactions between PARP and other regulatory proteins for this repressive effect.

To conclude, the interactions of PARP protein with the promoter of its own gene result in suppression of transcription. PARP binding to secondary structures in DNA may reflect a potential mechanism by which it is recruited to the gene promoter. Furthermore, our data suggest that a hierarchy of PARP function may exist under which transcriptional repression may be abrogated in response to DNA damage due to a higher affinity of PARP for DNA breaks and its dissociation from DNA following protein automodification (Fig. 6). This concept integrates PARP functions in DNA repair (a nick-protection mechanism) (4, 33) and in transcriptional control of gene(s) involved in immediate cellular response to ionizing radiation and DNA-damaging drugs. Although the evidence supporting such a mechanism is not yet available, it is conceivable that the sharing of components such as PARP by DNA repair and transcription allows both events to control cellular survival in response to ionizing radiation and DNA-damaging treatments. In support of this mechanism, PARP-dependent inhibition of transcription elongation by RNA polymerase II in undamaged cells and up-regulation of mRNA synthesis in response to DNA damage have been recently demonstrated both *in vitro* and *in vivo* (13). Studies testing this hypothesis are underway.

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Gene Therapy for Prostate Cancer by Targeting Poly(ADP-Ribose) Polymerase[1]

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ABSTRACT

Poly(ADP-ribose) polymerase (PARP) has strong affinity for DNA strand breaks and cycles on and off the DNA ends to allow DNA repair. A DNA-binding domain of PARP (PARP-DBD) acts as a dominant-negative mutant by binding to DNA strand breaks irreversibly and sensitizing mammalian cells to DNA-damaging agents. Therefore, expression of PARP-DBD in prostate carcinoma cells offers a strategy to achieve sensitization to genotoxic treatments. Toward this end, we developed recombinant plasmids expressing the PARP-DBD under the control of the 5′-flanking sequences of the human prostate-specific antigen (PSA) gene. Tissue specificity of PARP-DBD expression in human tumor cells was confirmed using the PSA-producing (LNCaP) and PSA-negative (PC-3) prostate cancer cells, as well as cells of nonprostate origin, Ewing's sarcoma (A4573 cells). LNCaP cells stably transfected with the PSA-regulated cDNA for PARP-DBD exhibit an androgen-dependent induction of PARP-DBD expression as determined by Western blotting, reverse transcription-PCR, and *in situ* immunofluorescence. Furthermore, we found that PARP-DBD sensitized LNCaP cells to DNA-damaging agents, such as ionizing radiation and etoposide. Androgen (R1881) -dependent stimulation of PARP-DBD expression resulted in a 2-fold growth inhibition in LNCaP cells as compared with controls, and an augmented apoptotic cell death in response to ionizing radiation or etoposide. Taken together, the plasmid vector developed in this study permits the expression of the human PARP-DBD in an androgen-inducible and PSA-dependent fashion, and sensitizes prostatic adenocarcinoma cells to DNA-damaging treatments. These results provide proof-of-principle for a novel therapeutic strategy for the treatment of prostate cancer.

INTRODUCTION

Prostate cancer is the most common malignancy in men, and it is the second most fatal cancer resulting in ~40,000 deaths annually in the United States. Whereas >80% of the tumors initially respond to androgen ablation, metastatic prostatic cancer inevitably progresses to an androgen-independent state. Once this happens, the disease is difficult to control, because hormonally independent tumors become resistant to additional hormonal manipulations as well as chemotherapy or radiotherapy (1).

PARP[3] is a zinc finger-containing protein, allowing the enzyme to bind either double- or single-strand DNA breaks. Numerous studies have shown that PARP is involved in a variety of biological functions, all of which are associated with breaking and rejoining of DNA strands, and plays a pivotal role in DNA damage repair (reviewed in Refs. 2–4). Recent advances in apoptosis research have identified PARP as one of the intracellular "death substrates," and have demonstrated that limited proteolysis of PARP by caspases is an early event or perhaps a prerequisite for the execution of programmed cell death in a variety of cells (5). The caspase-specific DEVD motif resides adjacent to the nuclear localization signal of the PARP protein. Cleavage of PARP at this site results in the separation of the two zinc finger DNA-binding motif in the NH_2 terminus of PARP from the automodification and catalytic domains located in the COOH terminus of the enzyme. Consequently, this cleavage excludes the catalytic domain from being recruited to the sites of DNA fragmentation during apoptosis and presumably disables PARP from coordinating subsequent repair of genome maintenance events. The biological function of the DNA-binding domain of PARP has been investigated by using stable cell lines expressing PARP-DBD protein (6). Data obtained from these experiments indicate that PARP-DBD expression in mammalian cells: (*a*) leads to *trans*-dominant inhibition of PARP; (*b*) has no effect on normal cell proliferation; and (*c*) sensitizes the cells to genotoxic agents and ionizing radiation. Exposure of the PARP-DBD-expressing cells to these DNA-damaging agents results in a marked reduction of cell survival, increased frequency of sister chromatid exchanges, inhibition of cell proliferation, and apoptosis induction (7). Thus, the DBD of PARP offers a potential for targeted sensitization of tumor cells to genotoxic agents and radiotherapy.

In the past few years several new approaches for treating advanced neoplasms have been proposed, including that of gene therapy. Differential expression of the desired gene product in the target tissue is central to the concept of gene therapy. One such approach is to use tissue-specific promoters to drive therapeutic genes. From this point of view, the promoter of the gene encoding the PSA represents a promising tool for prostate cancer-specific gene expression (8). Although low levels of PSA are detectable in the serum of men with normal prostates, PSA expression is increased in most patients with prostate cancer, regardless of tumor stage and hormone responsiveness. The promoter of the PSA gene has been cloned, and its two functional domains have been identified: a proximal promoter and a distal promoter, which can also function as an enhancer (9). Using LNCaP tumor xenografts in the nude mouse model it was demonstrated that the PSA promoter retained its tissue-specific properties *in vivo* (10). Furthermore, the PSA promoter was able to mimic the prostate-specific and androgen-regulated expression of the PSA gene in transgenic mice (11). Thus, the PSA promoter contains the features that are fundamental for the development of expression vectors for prostate-specific gene therapy: tissue specificity and androgen responsiveness.

The present study reports a novel approach for combination therapy that uses the tissue-specific (prostate) and DNA damage-specific (targeting the PARP function) gene therapy for prostate cancer. We describe the development of recombinant plasmids for expression of the DNA-binding domain of PARP under control of prostate tissue-specific promoter in PSA producing LNCaP prostate carcinoma cells. Our results show that enforced PARP-DBD expression significantly augments sensitivity of these cells to DNA-damaging treatments, presenting a novel strategy for gene therapy directed to prostate cancer.

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[3] The abbreviations used are: PARP, poly(ADP-ribose) polymerase; PARP-DBD, DNA binding domain of poly(ADP-ribose) polymerase; PSA, prostate-specific antigen; nt, nucleotide; aa, amino acid; RT-PCR, reverse transcription-PCR.

MATERIALS AND METHODS

Cell Lines and Tissue Culture. The androgen-responsive prostate carcinoma LNCaP and androgen-independent PC-3 cell lines were obtained from American Type Culture Collection and maintained by serial passage in DMEM supplemented with 10% fetal bovine serum. Cells subjected to androgen stimulation tests were maintained in medium with 10% charcoal-stripped fetal bovine serum for 7 days before the addition of synthetic androgen R1881 (Perkin-Elmer Life Science). Ewing's sarcoma cell line A4573 (kindly provided by Dr. Timothy Kinsella, University of Wisconsin, Madison, WI) was maintained in Eagle's MEM (Life Technologies, Inc.). All of the irradiations were performed in air, using a ^{137}Cs source in a JL Shepard MARK 1 laboratory irradiator at a dose rate of 3.85 Gy/min.

Plasmid Constructs. The 1.3-kb fragment that contains the upstream enhancer element (9, 10) of the PSA regulatory region (nt −745 to −2080) was cloned from human placenta using a PCR-generated probe corresponding to nts 1–200 of PSA cDNA. The PSA gene minimal promoter (nt −619 to +12) was obtained by performing PCR amplification using human placenta genomic DNA as a template, and set of primers 5′-GGTCTGGAGAACAAGGAGTG (upstream) and 5′-TCTCCGGGTGCAGGTGGTAA (downstream) designed according to the reported sequence data for the 5′ region of the human PSA gene (9). The 0.7-kb fragment of the human PARP cDNA encoding for DNA binding domain (aa 1–234) was PCR amplified using plasmid pCD12 containing cDNA for human PARP as a template and primers designed according to the reported sequence data (12). The human cDNA coding for the DNA-binding domain of PARP (5′-*Eco*RI-*Hind*III) was inserted into pcDNA 3.1 (−) expression vector at the *Eco*RI/*Hind*III restriction sites downstream of the human CMV promoter/enhancer. Subsequently, PARP-DBD was tagged at its COOH terminus with a sequence encoding four Flag-epitope tags (13). The resulting recombinant plasmid, pCMV-DBD/F, permits constitutive expression of human PARP-DBD under control of the CMV promoter. To express the human PARP-DBD under control of the PSA gene regulatory elements, the CMV promoter sequences were replaced with a 1336-bp *Xho*I-*Eco*RV fragment of PSA enhancer fused with an *Eco*RI fragment containing 662-bp sequence of PSA promoter. The resulting plasmid, pPSA(e/p)-DBD/F is designed to express human PARP-DBD in androgen-inducible and PSA-dependent fashion.

Transient DNA Transfections. DNA transfections were carried out using an activated-dendrimer (Superfect; Qiagen) as described (13). Cells (2×10^5) were transfected with 5 μg of pCMV-DBD/F or pPSA(e/p)-DBD/F plasmids using a ratio of DNA to Superfect reagent of 1:10. Assays for PARP-DBD expression were performed 48 h after transfection.

Stably Transfected LNCaP Cell Lines. Cells were transfected with pPSA(e/p)-DBD/F, pCMV-DBD/F, or with control, neomycin-resistant expression vector pΔCMV-DBD/F, respectively, using Superfect reagent (Qiagen). The G418-resistant colonies from each replicated experiment were pooled to form polyclonal cell populations and were routinely maintained in medium containing 300 μg/ml G418.

PARP-DBD Immunodetection. Logarithmically growing cells were washed twice with cold PBS and lysed at 4°C for 30 min in buffer: 0.5% Triton X-100, 0.5% NP40, 2 mM $NaOV_4$, 150 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl (pH 7.5), 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. For immunoprecipitation, cell lysates were normalized for protein content and incubated with anti-Flag M2 monoclonal antibody agarose affinity gel (Sigma), followed by Western blotting using polyclonal anti-PARP antibody (R&D System; 1:1000) directed against the aa 71–329 of PARP protein. The secondary antibody was donkey antigoat IgG conjugated to horseradish peroxidase (Santa Cruz, 1:2000). Signals were detected using the ECL system (Amersham). For *in situ* PARP-DBD immunodetection, LNCaP cells (PSA-DBD) were grown on poly-D-lysine-treated glass slides (Fisher Scientific). After induction of PARP-DBD expression by synthetic androgen R1881 (10 nM) for 24 h, cells were subjected to fixation with 3.7% paraformaldehyde and incubated for 30 min with anti-Flag M2 monoclonal antibody (Sigma; dilution 1:200). Washes were followed by 30-min incubation with Cy-5 conjugated secondary antibody (Jackson ImmunoResearch; dilution 1:200) in PBS, contained 10% donkey serum and 0.1% 300 Bloomgelatin. Transmitted and Cy5 fluorescence images were acquired using an IX 70 confocal microscope (Olympus, Melville, NY).

DNA Binding Assays. PARP and PARP-DBD affinity for DNA was assayed using double-stranded oligonucleotides coated onto magnetic beads. Briefly, 100 μg of streptavidin-coated Dynabeads (Dynal Biotech) were incubated with 120 pmols of 5′-biotinylated double-stranded pJα (5′-GTGAAAAAGGTGAAAAAG) oligonucleotides (14) in accordance with the manufacturer's instruction. Crude cell extracts in lP buffer were prepared from cells transiently transfected with pCMV-DBD/F or from parental LNCaP cells, and were normalized for protein content. Purified PARP protein (Alexis; specific activity 30 units/μg) or cell lysates were combined with pJα-affinity beads and incubated for 30 min with gentle agitation at room temperature. The protein-bound beads were separated using a magnetic separator (Dynal), and bound proteins were eluted with 30 μl of 1 M NaCl and subsequently analyzed by Western blotting using goat polyclonal anti-PARP antibody (R&D Systems). Recombinant human PARP and streptavidin-coated beads containing no DNA were used as positive and negative controls, respectively.

RT-PCR Analyses. RNA was isolated from cells using TRIzol Reagent (Life Technologies, Inc.) according to the manufacturer's protocol. The primers for human DBD-Flag fusion protein were: sense, 5′-ATCACCATCACCATCA-3′ and antisense, 5′-CCTTTATCGTCATCGT-3′. RT-PCR was performed using 2 mg of total cellular RNA and the ThermoScript RT-PCR System (Life Technologies, Inc.).

Proliferation and Apoptosis Assays. Growth characteristics of LNCaP cells were assayed by a colorimetric method using the tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2*H*-tetrazolium (Cell Titer 96 Aqueous Assay; Promega). Surface expression of phosphatidyl serine was determined by FITC-labeled annexin V staining (Trevigen) followed by analysis using a fluorescence-activated cell sorting Star Plus flow cytometer (Becton Dickinson). Apoptotic cells were defined as FITC positive and propidium iodide negative. Changes in the mitochondrial potential were analyzed using JC-1 DePsipher (Trevigen) reagent as suggested by manufacturer. Stained samples were analyzed at 488 nm argon laser by flow cytometry.

RESULTS AND DISCUSSION

Human PSA Promoter/Enhancer Drives Expression of the PARP-DBD in LNCaP Cells. This study is focused on the unique properties of the DNA-binding domain of PARP as a potent molecular sensitizer to DNA-damaging treatments. Data from several investigations demonstrated that genetically engineered PARP-DBD is critically involved in DNA damage repair by acting as a *trans*-dominant inhibitor of PARP activity and that its overexpression in mammalian cells sensitizes them to DNA-damaging treatments (7). In this study we isolated and cloned the fragment of human PARP cDNA encompassing the region (aa 1–234) that encodes two zinc fingers of the enzyme as well as the KKKSKK nuclear localization signal (PARP-DBD). Subsequently, we developed plasmid vectors to express human PARP-DBD as a Flag-fusion protein in human prostatic adenocarcinoma cells (LNCaP cell line) both constitutively and in androgen-dependent fashion (Fig. 1*A*). The recombinant plasmid, pCMV-DBD/F, permits constitutive expression of the PARP-DBD under control of the human CMV promoter. To achieve tissue-specific expression of the PARP-DBD in the androgen-sensitive LNCaP cells, we have constructed an expression vector, pPSA(e/p)-DBD/F, comprised of the coding region for the DNA-binding domain of PARP linked to 5′-flanked sequences (1.3 kb upstream enhancer and 0.6 kb minimal promoter) of the human PSA gene. The expression of the PARP-DBD Flag-fusion protein in LNCaP cells was confirmed in transient transfection assays (Fig. 1*B*). Immunoblot analysis of cell lysates revealed that exogenous PARP-DBD Flag fusion protein has a molecular mass of 31 kDa consistent with the length of corresponding cDNA, and is recognized by anti-Flag and anti-PARP antibodies. Functional activity of expressed PARP-DBD Flag-fusion protein was assayed in DNA binding reactions using double-stranded 5′-biotinylated oligonucleotides coupled to streptavidin-coated magnetic beads. These beads were used to recover DNA-binding proteins from LNCaP

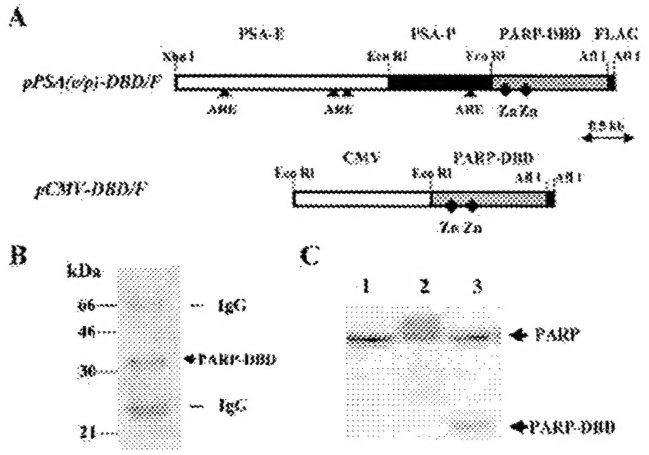

Fig. 1. *A*, a schematic representation of the recombinant constructs for constitutive (*pCMV-DBD*) and androgen-inducible (*pPSA-DBD*) expression of the human PARP-DBD in prostate cancer cells. *PSA-E*, 1.3 kb upstream PSA enhancer region; *PSA-P*, 0.6 kb minimal promoter of the human PSA gene. Relevant restriction enzyme sites, zinc fingers (*Zn*), and androgen response elements (*ARE*) are indicated. *B*, immunodetection of the PARP-DBD in LNCaP cells transiently transfected with pPSA(e/p)-DBD/F. *C*, detection of DNA-binding activity of wild-type PARP and PARP-DBD Flag-fusion proteins. Purified recombinant PARP (*1*) and cell extracts prepared from parental LNCaP cells (*2*) or LNCaP cells, transiently transfected with the pCMV-DBD/F (*3*) were bound to 5′-biotinylated double-stranded oligonucleotides coupled with streptavidin-coated magnetic beads. Proteins were eluted as described in "Materials and Methods" and analyzed by Western blotting using polyclonal anti-PARP antibodies.

cells transiently transfected with pCMV-DBD/F plasmid. We found (Fig. 1*C*) that both endogenous PARP and PARP-DBD fusion protein are captured effectively by DNA fragments, thus indicating that PARP-DBD retain its DNA-binding activity when expressed in LNCaP prostate carcinoma cells.

Androgen Responsiveness of the PARP-DBD Expression in LNCaP Cells. The 5′-regulatory sequences of the human PSA gene have been cloned and characterized (9). Deletion analysis of this region identified a minimal (core) promoter region (nt −320 to +12), strong upstream enhancer (nt −5824 to −3738), and the presence of down-regulating elements within the central region (nt −4136 to −541; Refs. 9, 10). Previous studies have identified the 5′-enhancer element linked to minimal core promoter of the human PSA gene as an effective combination of regulatory elements capable of driving the expression of reporter genes in PSA-producing prostate cancer cells both *in vitro* and *in vivo* (11, 15). Consequently, we developed and tested the construct, pPSA(e/p)-DBD/F, for its ability to drive the expression of PARP-DBD in a tissue-specific fashion, and its androgen responsiveness in prostate carcinoma cells.

The tissue specificity of PARP-DBD expression under control of the PSA promoter/enhancer was evaluated in transient transfection assays using the PSA-producing (LNCaP) and PSA-negative (PC-3) prostate cancer cells, as well as cells of nonprostate origin such as Ewing's sarcoma (A4573 cell line). We found that PSA enhancer/promoter-driven expression of the human PARP-DBD was immunodetectable only in PSA-producing LNCaP prostate carcinoma cells but not in PSA-independent cell lines (Fig. 2). Although more PSA-producing cell lines need to be tested to elaborate a PSA dependence of PARP-DBD expression, our data are consistent with findings reported previously that PSA promoter retains its tissue specificity both *in vivo* and *in vitro* (11, 15).

The 5′ flanking region of the human PSA gene contains several androgen-responsive elements and is responsible for the androgen-dependent expression of PSA in benign and malignant prostate cells. To evaluate whether the PSA promoter/enhancer constructs support androgen responsiveness of PARP-DBD expression, LNCaP carcinoma cells were stably transfected with PARP-DBD expression vectors, and established polyclonal LNCaP sublines (PSA-DBD and CMV-DBD) were subsequently subjected to analysis of PARP-DBD expression levels. Cells were grown in medium containing charcoal-stripped serum for 7 days followed by incubation for 24 h in the absence or presence of the synthetic androgen, R1881 (0–10 nM). Western blot analysis and RT-PCR were performed to evaluate androgen-regulated expression of the human PARP-DBD in LNCaP cells (Fig. 3). Parental LNCaP cells and the LNCaP cell subline (CMV-DBD) were used as negative and positive controls, respectively, for PARP-DBD expression levels in these experiments. We found that exposure of PSA-DBD cells to androgen (R1881) resulted in dose-dependent stimulation of PARP-DBD expression at levels of mRNA (Fig. 3*B*) and protein (Fig. 3*A*). No notable changes in the PARP-DBD expression levels have been observed in control cell lines exposed to R1881 at doses up to 10 nM (data not shown). Androgen-dependent regulation of PARP-DBD expression in PSA-DBD prostate carcinoma cells was additionally confirmed by *in situ* immunodetection of the PARP-DBD-Flag fusion protein using fluorescence microscopy (Fig. 3*C*). These data indicate that the pPSA(e/p)-DBD/F recombinant vector allows expression of functionally active DBD of PARP *in vitro* and that the androgen-dependent expression is specific to PSA-producing prostate carcinoma cells.

PARP-DBD Expression Sensitizes LNCaP Cells to DNA Damage. The PARP-DBD fragment acts as a *trans*-dominant inhibitor of PARP activity by competing with endogenous wild-type PARP for DNA strand breaks (6). Furthermore, using atomic force microscopy we have demonstrated recently that PARP-DBD binds to broken DNA strands irreversibly (16), making them inaccessible to DNA repair enzymes. These data suggest that forced expression of the PARP-DBD can impair the function of endogenous PARP in cellular responses to DNA damage leading to accumulation of sustained lesions in the genome, thereby overcoming cellular resistance to radio- and chemotherapeutic intervention. In support of this suggestion, the sensitization of the DBD-expressing mammalian cells to ionizing radiation and DNA-damaging agents has been demonstrated recently (7).

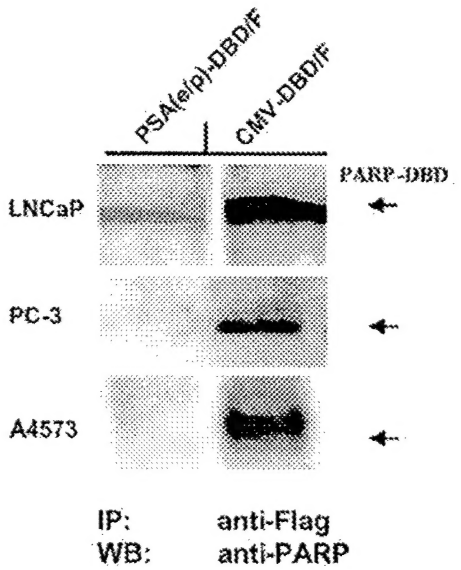

Fig. 2. PARP-DBD expression in PSA-producing and PSA-negative cells. PSA-positive (*LNCaP*) or PSA-insensitive (*PC-3*) prostate cell lines, and nonprostate (Ewing's sarcoma) A4573 cell line were transiently transfected with pPSA(e/p)-DBD/F or pCMV-DBD/F plasmids. Cells extract were prepared 48 h after transfection and PARP-DBD Flag-fused protein was immunodetected as described in "Materials and Methods."

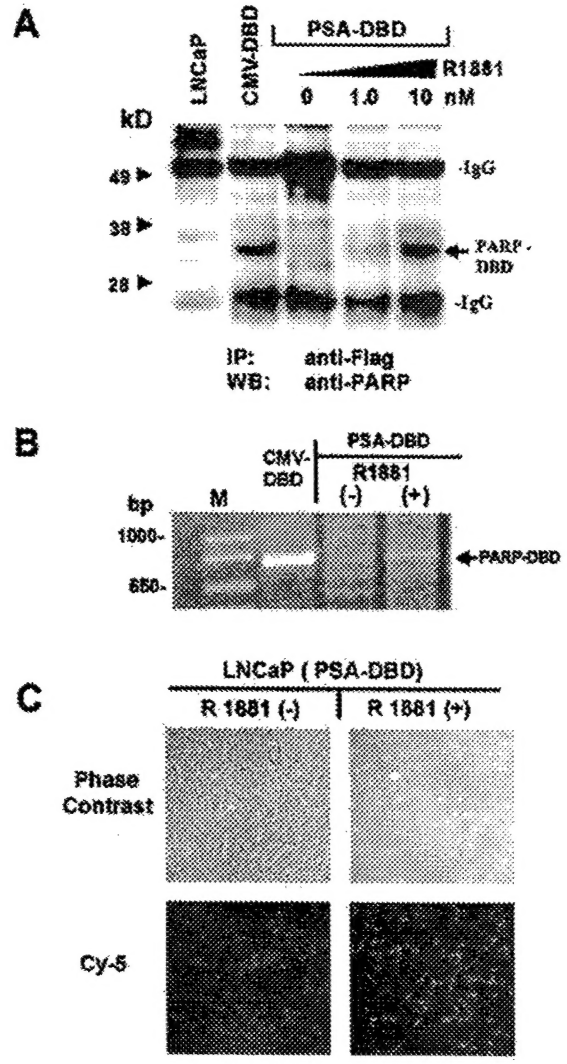

Fig. 3. Androgen responsiveness of the PARP-DBD expression in LNCaP cells. *A*, LNCaP cells were stably transfected with vectors for PARP-DBD expression under the control of CMV (*CMV-DBD* cell subline) or PSA gene (*PSA-DBD* cell subline) promoters. Cells were maintained for 7 days in medium containing 10% charcoal-stripped fetal bovine serum followed by induction of the PARP-DBD expression with R1881 for 24 h. Immunodetection of the PARP-DBD-Flag fusion protein in parental LNCaP cells, and PARP-DBD expressing LNCaP sublines was performed as described in "Materials and Methods." The migration of the PARP-DBD is indicated on the *right*. *B*, RT-PCR analysis of mRNA for PARP-DBD-Flag fused protein in stably transfected CMV-DBD and PSA-DBD LNCaP cells. PARP-DBD expression in PSA-DBD cells was induced with R1881 as above. Specific RT-PCR products are indicated on the *right*, and molecular weight markers (*M*) are shown on the *left*. *C, in situ* PARP-DBD immunodetection in LNCaP (*PSA-DBD*) cells. After induction of PARP-DBD expression by synthetic androgen R1881 (10 nM) for 24 h, cells were immunostained for PARP-DBD-Flag fusion protein using anti-Flag M2 monoclonal antibody and Cy-5 conjugated secondary antibody as described in "Materials and Methods." Transmitted (*phase contrast*) and Cy5 (*red fluorescence*) images were acquired using IX 70 confocal laser scanning microscope (Olympus).

To investigate whether PARP-DBD would increase the susceptibility of human prostate carcinoma to DNA-damaging treatments, the expression of PARP-DBD in LNCaP (PSA-DBD) cells was induced by R1881, and cells were subsequently exposed to ionizing radiation or etoposide (VP-16). We found that androgen (R1881) -dependent stimulation of PARP-DBD expression significantly enhanced (at least 2-fold) growth inhibition of PSA-DBD cells in response to DNA damage, compared with control cells (Fig. 4*A*). This inhibition can be attributed to PARP-DBD expression in LNCaP cells (Fig. 3) rather then to the presence of androgen in the growth medium. In fact, other studies have shown that androgens are potent stimulators of LNCaP cells growth *in vitro* (17).

We next examined whether the PARP-DBD-mediated sensitization of LNCaP cells to DNA damage is attributable to an increased rate of apoptosis. Quantitative measurements of cell death were carried out using annexin V-propidium iodide staining and mitochondrial depolarization assays. Previous studies (18) show that LNCaP cells are highly resistant to ionizing radiation and fail to activate classical apoptotic pathways in response to DNA-damaging treatments. In agreement with these findings, we found that parental LNCaP cells as well as the uninduced PSA-DBD cell subline exhibit only marginal levels of cell death after exposure to ionizing radiation or etoposide (Fig. 4). When PARP-DBD expression was induced by R1881, irradiated or etoposide-treated LNCaP (PSA-DBD) cells showed significantly (>2-fold) increased staining for annexin V (Fig. 4*B*) and depolarization of mitochondrial membrane (Fig. 4*C*) within 24 h of treatment. These data indicate that perturbation of PARP function via enforced expression of its dominant-negative mutant (PARP-DBD) results in enhanced sensitivity of prostate cancer cells to DNA-damaging treatments. Considering the fact that androgens block apoptosis in LNCaP cells triggered by diverse agents, including ionizing

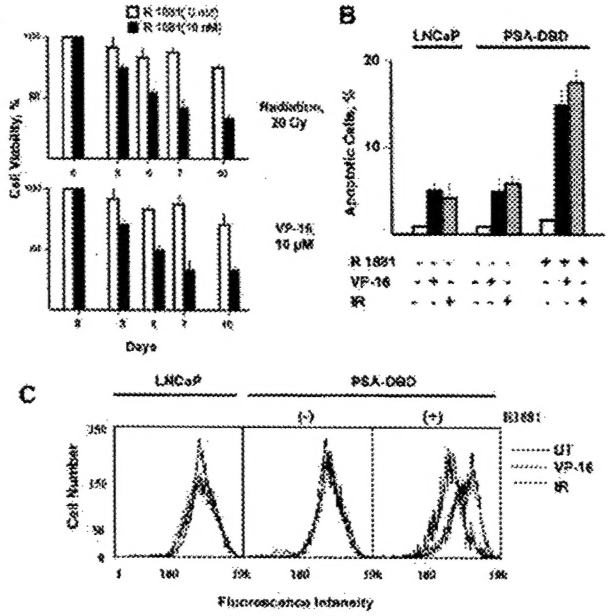

Fig. 4. PARP-DBD sensitizes human prostate cancer cells to ionizing radiation and etoposide. *A*, PARP-DBD expression enhances DNA damage-induced growth inhibition in prostate carcinoma cells. LNCaP (*PSA-DBD*) cells were maintained in medium containing 10% charcoal-stripped fetal bovine serum in presence (■) or absence (□) of synthetic androgen R1881 (10 nM) before irradiation (20 Gy) or treatment with etoposide (10 μM). Viable cells were measured by an 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2*H*-tetrazolium assay at indicated times, and results are expressed as a percentage of mock-treated control ($n = 4$); *bars*, ±SD. *B*, effect of PARP-DBD expression on annexin staining in LNCaP cells after DNA-damaging treatments. PSA-DBD cells were maintained in absence or in presence of R1881 (10 nM) for 24 h before irradiation (*IR*; 20 Gy) or treatment with etoposide (10 μM; *VP-16*). Annexin V binding activity was determined in parental LNCaP and PSA-DBD cells by flow cytometry 24 h after treatments. Apoptotic cells are defined as annexin V-positive cells and are expressed as percentage of total cell number in sample analyzed on FACSscan flow cytometer. Data presented are the mean values determined from triplicate experiments; *bars*, ±SD. *C*, effect of PARP-DBD expression on changes of mitochondrial membrane potential in LNCaP cells after DNA-damaging treatments. After treatments (24 h), untreated controls (*UT*), irradiated (*IR*; 20 Gy), or etoposide-treated (10 μM; *VP-16*) cells were stained with JC-1 "DePsipher" reagent and analyzed by flow cytometry. Mitochondrial potential breakdown in dying cells results in accumulation of green fluorescent JC-1 monomers, which, in turn, is reflected by an increase of green fluorescence events. Representative data (of three independent experiments) are shown.

radiation (19), our observations suggest that overexpression of the PARP-DBD augments apoptotic pathways in these cells in an androgen-independent fashion. Additional investigations are required to elucidate the mechanisms for PARP-DBD-mediated sensitization of LNCaP cells to DNA damage, as well as the enhanced apoptotic responses in DBD-expressing prostate cancer cells. The studies addressing these questions are currently underway.

Although many prostate cancer cells are deficient in DNA mismatch repair, they are resistant to ionizing radiation and DNA-damaging drugs. Therefore, targeting molecular components that are critically involved in maintenance of genome stability is a promising approach directed at overcoming intrinsic tumor cell resistance to DNA-damaging treatments. From this point of view, the strategy described here represents a novel starting point for the design of PARP-based molecular therapies targeting prostate cancer *in vivo*. First, this approach uses tissue-specific (prostate carcinoma) and treatment-specific (DNA damage) gene therapy for prostate cancer. Next, to avoid the potential side effects due to expression of PSA in tissues other than prostate, tumor cells are targeted using an agent that is not functionally active in the absence of massive DNA damage and, therefore, would not be toxic to cells outside of the irradiated volume or pose a genetic risk to the patient. Furthermore, PARP-DBD-mediated cell death is independent of cell proliferation states because both nondividing cells and rapidly proliferating cancer cells cannot survive the massive accumulation of long-lived damage in the genome (20). Thus, targeting tumor cells with the PARP-DBD can be beneficial especially for the control of prostate cancer, because prostate cancers usually grow very slow. These properties of the PARP-DBD are in marked contrast to conventional chemotherapeutic drugs, which primarily target proliferating cells. In summary, the plasmid vector developed in this study permits the expression of the human PARP-DBD in an androgen-inducible and PSA-dependent fashion, and sensitizes prostatic adenocarcinoma cells to DNA-damaging treatments. These results provide a proof-of-principle for a novel therapeutic strategy to control prostate cancer.

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